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(54) Selection marker gene free recombinant strains, a method for obtaining them and the use of these strains

Selektionmarker-genfreie rekombinante Stämme: Verfahren zur ihrer Herstellung und die Verwendung dieser Stämme

Souches récombinantes dépourvues de marqueurs de sélection: procédé pour leur obtention et utilisation de ces souches

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(73) Proprietor: **DSM N.V.**
6411 TE Heerlen (NL)

(72) Inventors:
- **Selten, Gerardus Cornelis Maria**
  NL-2651 HZ Berkel en Rodenrijs (NL)
- **van Gorcom, Robertus Franciscus Maria**
  2622 DE Delft (NL)
- **Swinkels, Bart Willem**
  NL-2611 MX Delft (NL)

(74) Representative:
Matulewicz, Emil Rudolf Antonius, Dr. et al
DSM N.V.
Patents & Trademarks Department
Office Delft 600-0240
P.O. Box 1
2600 MA Delft (NL)

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WO-A-93/01283

- **MOLECULAR & GENERAL GENETICS, vol.236, no.1, December 1992 pages 121 - 124 CARL T. YAMASHIRO ET AL. 'A dominant selectable marker that is meiotically stable in Neurospora crassa: the amdS gene of Aspergillus nidulans'**
- **CURRENT GENETICS, vol.11, 1987 pages 639 - 641 RAJ K. BERI ET AL. 'Transformation of Penicillium chrysogenum using the Aspergillus nidulans amdS gene as a dominant selective marker'**

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Description

Technical field

[0001] The present invention discloses selection marker gene free recombinant filamentous fungal strains, a method for obtaining these strains and the use of these strains. Furthermore, the method of the present invention is used for performing strain improvement.

Background of the invention

[0002] There is an increasing social concern about the use of recombinant DNA technology. One of the promising application areas of recombinant DNA technology is strain improvement. Starting from the early days of fermentative production processes there has been a demand for the improvement of the productivity of the strains used for production.

[0003] Classical strain improvement programs for industrially employed microorganisms are primarily based on random mutagenesis followed by selection. Mutagenesis methods have been described extensively; they include the use of UV light, NTG or EMS as mutagens. These methods have been described extensively for example in "Biotechnology : a comprehensive treatise in 8 vol." Volume I, Microbial fundamentals, Chapter 5b, Verlag Chemie GmbH, Weinheim, Germany.

[0004] Selection methods are generally developed around a suitable assay and are of major importance in the discrimination between wild type and mutant strains.

[0005] It has turned out that these classical methods are limited in their potential for improvement. Generally speaking consecutive rounds of strain improvement yield diminishing increases in yield of desired products. This is at least partially due to the random character of the mutagenesis methods employed. Apart from desired mutations these methods also give rise to mutations which are undesirable and which may negatively influence other characteristics of the strains.

[0006] In view of these drawbacks it can be understood that the use of recombinant DNA methods was hailed as a considerable improvement. In general, recombinant DNA methods used in strain improvement programs aim at the increased expression of desired gene products.

[0007] The gene products may be proteins that are of interest themselves, on the other hand it is also possible that the encoded gene products serve as regulatory proteins in the synthesis of other products.

[0008] Strains can be improved by introducing multiple copies of desired protein encoding genes into specific host organisms. However, it is also possible to increase expression levels by introducing regulatory genes.

[0009] Genes are introduced using vectors that serve as vehicles for introduction of the genes. Such vectors may be plasmids, cosmids or phages. The vector may be capable of expression of the genes in which case the vector generally is self-replicating. The vector may however also only be capable of integration. Another characteristic of the vector is that, when the expression product cannot be selected easily based on altered phenotypic properties, the vector is equipped with a marker that can easily be selected for.

[0010] Vectors have not been isolated from all known microorganisms either since no vector could be found in the organism or since available vectors from other organisms could be used with little or no modification. The same applies to selection marker genes.

[0011] Widespread use and the subsequent spreading of specific marker genes has recently become debatable. This is especially due to the finding that the use of antibiotics and antibiotic selection markers gives rise to an undesired spread of strains that have become antibiotic resistant. This necessitates the continued development of novel ever more potent antibiotics.

[0012] It is therefore not surprising that there is a general tendency in large scale production to use recombinant microorganisms containing no antibiotic resistance genes or more generally as little as possible of foreign DNA.

[0013] Ideally the transformed microorganism would contain only the desired gene(s), fragments thereof or modifications in the gene and as little as possible or no further remnants of the DNA used for cloning.

[0014] WO 93/01283 discloses recombinant marker gene free plant cells from which marker genes are excised using the bacteriophage P1 cvr/lox system.

Summary of the invention

[0015] The present invention discloses a selection marker gene that can easily be deleted again from the recombinant host organism. The deletion of the said marker gene is based on dominant selection.

[0016] The marker is used filamentous fungi.

[0017] The advantageous activity of the selection markers used herein is based on the following two step principle:

a) the gene is integrated into the genome of the host organism and recombinant cells are selected,
b) the transformed cell is grown on a substrate, which is converted by the marker gene encoded activity to a product that is lethal to the cell.

[0018] Selected cells will be recombinant and will have deleted the selection marker gene.

[0019] In general terms the present invention discloses filamentous fungal cells, that have a modification in the genome characterized in that the alteration is introduced using the amdS gene or the cDNA derived therefrom.

[0020] An example of a selection marker gene that can be used in this way is the acetamidase gene. Preferably, this gene is obtainable from filamentous fungi, more preferably from Aspergilli, most preferably from Aspergillus nidulans.

[0021] The invention further shows the introduction, deletion or modification of desired heterologous or homologous genes or DNA elements in the filamentous fungus using the acetamidase (amdS) gene as a marker. Subsequently the amdS gene is deleted. Preferably, the amdS and the desired genes are introduced site-specifically.

[0022] The invention discloses a vector containing:

a) a desired DNA fragment destined for introduction into the host genome,
b) optionally a DNA sequence that enables the vector to integrate (site-specifically) into the genome of the host strain,
c) a gene encoding an acetamidase (e.g. the amdS gene from A.nidulans) between DNA repeats.

[0023] The invention further discloses filamentous fungi transformed with the said vector.

[0024] The invention further discloses selection marker gene free recombinant filamentous fungi.

[0025] Specifically, the invention discloses filamentous fungi containing site-specifically introduced genes without any further foreign DNA being present. The method is therefore also suited for repeated modifications of the host genome, e.g. the sequential introduction of multiple gene copies at predetermined loci.

[0026] The invention provides a method for obtaining selection marker gene free recombinant filamentous fungal strains comprising the following steps:

a) integration into the genome of the strain of a desired DNA fragment and a selection marker,
b) selection of the recombinants,
c) deletion of the selection marker preferably using internal recombination between selection marker flanking repeats,
d) counter-selection based on the absence of the selection marker.

[0027] Although this is the preferred method for obtaining selection marker gene free recombinant filamentous fungal strain, the invention also provides modifications of this method, for example:
The desired DNA fragment and the selection marker may be present on two different DNA molecules which are co-transformed. The selection marker does not necessarily integrate into the genome of the strain but may be present on an episomal DNA molecule which can be cured.

[0028] The present invention further illustrates that this marker gene can be deleted from the genome of the transformed organisms without leaving a trace i.e. DNA used for cloning.

[0029] The invention discloses also the use of the amdS gene for deleting a desired gene from the chromosome of a filamentous fungal 'host' organism. In specific embodiments the following strains are employed Aspergilli, Trichoderma, Penicillium.

[0030] The method of the present invention provides recombinant strains with genomic modifications obtained by repeating the procedure with the same or other vectors.

Brief description of the drawings

Abbreviations used in the figures:

Restriction enzymes and restriction sites:

[0031]

A = ApaI; Ba = BamHI; B = BglII; Bs = BssHII; E = EcoRI;
H = HindIII; K =KpnI; N = NdeI; N = NotI; Ps = PstI;
P = PvuII; Sa = SalI; Sc = ScaI; S = SmaI; Sn = SnaBI;
Spe = SpeI; Sp = SphI; Ss = SstII; Xb = XbaI; X = XhoI.

Other:

[0032] T. = LAC4 terminator sequence

Figure 1: shows the restriction map of plasmid pamdS-1. This plasmid contains the cDNA of the amdS gene from A.nidulans.

Figure 2: shows schematically the marker gene free deletion of the glaA locus from A.niger using the gene replacement vector pGBDEL4L. The essential part of the gene replacement vector pGBDEL4L contains the amdS gene under control of the gpdA promoter cloned between repeats (3'-non-coding region of the glaA gene).

Figures 3-9: show schematically the construction pathway of pGBDEL4L as further outlined in Example 1.

Figure 10 A. KpnI digests of pGBDEL4L transformants #41 (lane 1), #24 (lane 2), #23 (lane 3) and #19 (lane 4) and the host strain A.niger CBS 513.88 (lane 5) and BamHI digests of pGBDEL4L transformants #41 (lane 6), #24 (lane 7), #23 (lane 8), #19 (lane 9) and the host strain A.niger CBS 513.88 (lane 10), probed with ^{32}P-labelled glaA promoter fragment and xylanase probe.
B. KpnI digests of GBA-102 (lane 1) and the GBA-102 strains after fluoracetamide selection: GBA-107 (lane 2) and GBA-108 (lane 3) and BamHI digests of GBA-102 (lane 4) and the GBA-102 strains after fluoracetamide selection: GBA-107 (lane 5) and GBA-108 (lane 6), probed with ^{32}P-labelled glaA promoter fragment and xylanase probe.

Figure 11: A. Schematic presentation of BamHI and KpnI fragment lengths of the wild-type glaA locus in Aspergillus niger CBS 513.88.
B. Schematic presentation of BamHI and KpnI fragment lengths of the truncated glaA locus in transformant #19 (= GBA-102). C. Schematic presentation of BamHI and KpnI fragment lengths of the truncated glaA locus in GBA-102 transformants after removal of the amdS gene (=GBA-107 and GBA-108).

Figure 12: A: shows schematically the integration of the glaA gene into the 3' non-coding region of truncated glaA locus of A.niger GBA-107.
B: shows the result of the internal recombination between the 3' glaA repeats, flanking the amdS gene.

Figures 13-24: show schematically the construction pathway of the integration vector pGBGLA30 as further outlined in Example 2.

Figure 25: BglII digests of pGBGLA30 transformants #107-9 (lane 1), #107-7 (lane 2) and #107-5 (lane 3), the host strain A.niger GBA-107 (lane 4) and the parental strain A.niger CBS 513.88 (lane 5) and KpnI digests of pGBGLA30 transformants #107-9 (lane 6), #107-7 (lane 7) and #107-5 (lane 8), the host strain A.niger GBA-107 (lane 9) and the parental strain A.niger CBS 513.88 (lane 10), probed with ^{32}P-labelled glaA promoter fragment.

Figure 26: A: Schematic presentation of the KpnI and BglII fragment lengths of the wild-type glaA locus in Aspergillus niger CBS 513.88.
B: Schematic presentation of the KpnI and BglII fragment lengths of the truncated glaA locus in Aspergillus niger GBA-107.
C: Schematic presentation of the KpnI and BglII fragment lengths of the truncated glaA locus with a single copy pGBGLA30 integrated into the glaA 3'-non-coding region as in transformants #107-5 (= GBA-119) and #107-9 (= GBA-122).
D: Schematic presentation of the KpnI and BglII fragment lengths of the truncated glaA locus in GBA-119 and GBA-122 transformants after removal of the amdS gene (= GBA-120, GBA-121, GBA-123 and GBA-124).

Figure 27: A: BglII digests of A.niger CBS 513.88 (lane 10), GBA-107 (lane 9), GBA-119 (lane 8) and the GBA-119 strains after fluoracetamide selection: #AG5-7 (= GBA-120) (lane 5), #AG5-5 (= GBA-121) (lane 6) and #AG5-6 (lane 7); GBA-122 (lane 4) and the GBA-122 strains after fluoracetamide selection: #AG9-1 (= GBA-123) (lane 3), #AG9-2 (lane 2) and #AG9-4 (= GBA-124) (lane 1), probed with ^{32}P-labelled 3"glaA non-coding fragment.
B: KpnI digests of A.niger CBS 513.88 (lane 10), GBA-107 (lane 9), GBA-119 (lane 8) and the GBA-119 strains after fluoracetamide selection: #AG5-7 (= GBA-120) (lane 5), #AG5-5 (= GBA-121) (lane 6) and #AG5-6 (lane 7); GBA-122 (lane 4) and the GBA-122 strains after fluoracetamide selection:

#AG9-1 (= GBA-123) (lane 3), #AG9-2 (lane 2) and #AG9-4 (= GBA-124) (lane 1), probed with ^{32}P-labelled 3" glaA non-coding fragment.

Figure 28: shows schematically the construction pathway of pGBGLA50.

Figures 29-33: show schematically the construction pathway of pGBGLA53.

Detailed description of the invention

[0033] The present invention discloses the use of a marker for preparing selection marker gene free recombinant filamentous fungal host strains. The selection marker gene can be used on an episomal DNA vector. However, in the present invention, the marker gene is preferably integrated into the genome of the filamentous fungal host strain. The advantage of the selection marker of the present invention is that it is a non-antibiotic dominant selection marker. Another advantage of the selection marker of the present invention is that it can be easily deleted from the transformed filamentous fungal host organism. The deletion of the marker is based on dominant selection. As such the selection marker of the present invention is a dominant and bi-directional selection marker. To our knowledge it is the only selection-marker available which is bidirectional and dominant in both directions.

[0034] In the present description we use the term selection marker gene'. With this term we mean the DNA coding for the marker protein in a functional form irrespective of whether it is the actual gene or the cDNA derived therefrom. The gene or cDNA is used dependent on the host organism and the expected splicing problems.

[0035] In the present invention we use the term 'vector'. By this is intended any DNA molecule that can be introduced into a selected filamentous fungal host irrespective of whether the vector integrates into the genome of the filamentous fungal host cell or remains episomal. The vector contains a selectable marker gene functional in the selected host or can be co-transformed with another DNA molecule containing such a selection marker gene.

[0036] The present description uses the term 'desired heterologous or homologous genes or DNA fragments'. By this is intended a DNA fragment that may be obtained from the filamentous fungal host strain or from another species or strain. The desired DNA fragment may contain any genetic element, parts thereof or combinations thereof, such as a gene (coding part or complete locus), a cDNA, a promoter, a terminator, an intron, a signal sequence, any regulatory DNA sequence or recognition sequence of DNA-binding proteins. The fragment may also be a DNA sequence that has been modified i.e. contains one or more nucleotide alterations (e.g. insertions, deletions, substitutions).

[0037] The present description further uses the term 'introduction' of a desired gene or DNA fragment. By this is intended an insertion deletion, substitution of desired DNA sequences in a selected filamentous fungal host cell.

[0038] The term 'genetic modification' used in the present invention refers to any modification of DNA sequences in a selected filamentous fungal host cell which is the result of the introduction of any one of the above mentioned desired DNA fragments into the host cell, preferably by transformation or co-transformation.

[0039] In general all these genetic modifications can be performed using the method of the present invention with subsequent deletion of the selection marker gene. Due to the fact that the recombinant filamentous fungal strain containing such a genetic modification does not contain the selection marker gene, the procedure of the present invention can be repeated, so that the modifications suggested above can be combined in the recombinant filamentous fungal strain. Ultimately, the procedure of the present invention can be used repeatedly up to the point that a recombinant filamentous fungal strain is obtained from which all undesired activities have been removed by deletion or inactivation of the corresponding genetic elements and which contains the desired acivities at the desired levels by sequential introduction of the corresponding desired DNA fragments at desired copynumbers and preferably at desired and defined loci.

[0040] The A. nidulans acetamidase (amdS) gene allows A. nidulans to grow on acetamide as the sole N-source. For microorganisms that lack the possibility or only have a very limited capacity to use acetamide as the sole N-source the acetamidase gene can in principle be used as a selection marker provided that acetamide is taken up by the cells. The amdS gene has successfully been employed as a marker gene in Aspergilli (Kelly and Hynes (1985) EMBO J. 4, 475-479; Christensen et al. (1988) Bio/technology 6, 1419-1422), Penicillium (Beri and Turner (1987) Curr. Genet. 11, 639-641) and Trichoderma (Pentillä et al. (1987) Gene 61, 155-164), and Neurospora (Yashimoto et al. (1992) Mol. Gen. Genet. 236, 121-124).

[0041] The amdS gene from A.nidulans is capable of converting acetamide to ammonia and acetic acid. This property enables A.nidulans to grow on a medium containing acetamide as the sole N-source or C-source.

[0042] Another property of the amdS gene is that it is also able to convert fluoracetamide to ammonia and fluoracetic acid. Fluoracetic acid however is toxic to the cell. It allows the production of marker gene free recombinant filamentous fungal strains. The fluoracetamide converting property enables the counter-selection of transformed filamentous fungal cells. The amdS gene is introduced into the filamentous fungal host strain and integrated into the genome through homologous recombination. The transformed filamentous fungal strains are selected on a medium containing acetamide as the sole N-source. Subsequently the selected strains are grown on a medium containing fluoracetamide and

urea (or other preferably defined N-sources) as the sole N-sources. The surviving filamentous fungal strains will have deleted the amdS gene.

[0043] The present invention uses the A.nidulans amdS gene as acetamidase marker gene. The relevant properties provided by the acetamidase encoded by the A.nidulans amdS gene, i.e. the ability to hydrolyse acetamide into ammonia and acetate as well as the ability to liberate fluoracetic acid from fluoracetamide, can also be provided by acetamidases from other sources. Use of an acetamidase marker gene is therefore not restricted to the A.nidulans amdS gene but includes any DNA sequence encoding a functional acetamidase.

[0044] The frequency of marker deletion is substantially increased by increasing the capacity of the gene for intrachromosomal homologous recombination. To achieve this the amdS gene is preferably placed between DNA repeats. These repeats are not necessarily both present in the vector but may also be created by a single cross-over integration. Alternatively, one may omit flanking repeats and rely on other mechanisms for removal or inactivation of the marker gene. In that case, however, the outcome may be less predictable and may not result in removal but rather in mere inactivation of the marker gene.

[0045] The vector may be constructed in such a way that, after deletion of the marker gene, no extraneous foreign DNA (except the DNA of interest) remains in the chromosome of the filamentous fungal host strain. The invention discloses a vector comprising:

a) a desired DNA fragment destined for introduction into the filamentous fungal host genome,
b) optionally a DNA sequence that enables the vector to integrate (site-specifically) into the genome of the filamentous fungal host strain,
c) a gene encoding an acetamidase (e.g. the amdS gene from A.nidulans) between DNA repeats.

[0046] Identical results may be obtained when the DNA-fragment destined for introduction into the filamentous fungal host genome and the selectable marker gene (e.g. the acetamidase gene) are present on two different DNA molecules which are co-transformed, in which case the DNA molecule containing the selectable marker does not necessarily integrate into the filamentous fungal host genome but may be present on an episomal DNA molecule which can be cured.

[0047] The sequences used for integration as mentioned under b) are used if site-specific (or better locus specific) integration is desired. If such a sequence is not present the vector nevertheless may integrate into the genome. This does not influence the ability to delete the selection marker gene.

[0048] The dominant counter-selection described above can be employed in the development of industrial filamentous fungal production strains in various ways. The use of a dominant selection marker is especially advantageous in the development of improved filamentous fungal production strains due to the fact that these strains are often diploid or polyploid.

[0049] The vector used for integration of the amdS gene preferably contains another gene of interest. The invention thus further enables the introduction of desired foreign or homologous genes or DNA elements in the filamentous fungal host organisms of choice using the amdS gene as a marker. Subsequently the amdS gene is deleted. Preferably, the amdS and the desired genes or DNA elements are introduced site-specifically, whereafter the amdS gene is deleted.

[0050] Specifically, the invention discloses filamentous fungal organisms containing site-specifically introduced genes without any further foreign DNA being present. The invention is used for integration of multiple copies of a desired gene or a DNA element at predetermined genomic loci.

[0051] The invention provides a method for obtaining selection of marker gene free filamentous fungal recombinant strains comprising the following steps:

- integration of a desired gene or DNA element and a selection marker by homologous recombination between sequences incorporated in an expression cassette and sequences on the filamentous fungal host chromosome,
- selection using the selection marker gene that is dominant,
- deletion of the selection marker gene using selection marker gene flanking regions,
- selection based on the absence of the selection marker gene (counter-selection).

[0052] The present invention further shows that this marker gene can be deleted from the chromosomes of the transformed filamentous fungal organisms without leaving a trace i.e. DNA used for cloning. Moreover, the invention also shows that similar if not identical results can be obtained when the desired gene or DNA element and the selection marker are present on two different DNA molecules which are co-transformed.

[0053] Finally the invention discloses the use of the amdS gene for deleting a desired gene from the chromosome of a filamentous fungal 'host' organism.

[0054] In view of the above, the method of the present invention is ideally suited for, but not limited to the cloning and expression of genes coding for proteins used in food, feed or pharmaceutical applications or genes involved in

biosynthesis of antibiotics and other bio-active compounds, i.e. recombinant proteins and/or hosts-organisms that are subject to strict registration requirements.

[0055] Examples of such proteins are well known in the art and include chymosin, phytase, xylanases, amylases, cellulases and hemicellulases, cytokines and other pharmaceutical proteins, etc.

[0056] The same method is employed for deletion of genes coding for proteins that influence production levels of desired proteins again without leaving a marker gene in the genome. Such proteins include proteases which actively digest the desired products that are highly expressed in the host strain and that therefore have a reduced potential of producing and or secreting the desired proteins. A preferred method for the deletion of a given gene would use a DNA construct containing the following elements in a 5' to 3' order: sequences 5' of the gene to be deleted, directly fused to sequences 3' of the gene to be deleted, followed downstream by a functional selection marker gene (preferably an acetamidase gene), followed downstream by again sequences 3' of the gene to be deleted. In this case both sequences 3' of the gene to be deleted are chosen such that they form repeats flanking the selection marker gene. Transformation of this DNA construct and subsequent replacement of the chromosomal copy of the gene to be deleted by the DNA construct with cross-over points in the sequences 5' and 3' of the gene to be deleted results in deletion of the given gene. Subsequent intrachromosmal recombination between the repeats flanking the selection marker gene and counter-selection for these recombinants finally results in a selection marker free filamentous fungal strain with the given gene deleted. The DNA construct used for this deletion can be constructed such that no foreign DNA or other traces of the genetic modification are left in the strain carrying the deletion.

[0057] The invention discloses selection marker gene free recombinant filamentous fungal microorganisms. Such microorganisms can be organisms that, after the use of the disclosed technology, contain an extra copy of a desired gene (either homologous or s heterologous). Such filamentous fungal microorganisms can be re-transformed over and over by sequential application of the same technology to insert or delete additional copies of the same or other gene (s) of interest.

[0058] The filamentous fungal microorganisms may also be characterized in that they have (a) predetermined gene (s) deleted or altered in any desired way.

[0059] The method of the present invention makes possible the fine-tuning of the production of desired proteins. This possibility is based on the ease with which repeated rounds of insertion and deletion can be performed. The method makes possible the insertion or deletion of a desired number of gene copies. Thus the proteins are produced in desired amounts and in desired ratios. This is especially useful for the production of mixtures of proteins or enzymes.

[0060] Whereas it is known that the acetamidase gene is capable of conversion of acetamide as the sole N-source in Aspergillus it is here shown that the acetamidase gene is easily deleted from the genome of transformed Aspergilli. To achieve this the amdS gene is cloned between direct repeats. In principle any direct repeat which allows for internal recombination can be employed. In the present examples this is demonstrated by cloning the amdS gene between 3' amyloglucosidase (glaA) non-coding DNA sequences.

[0061] It is shown that the amdS gene can be integrated and deleted upon plating on medium containing fluoracetamide and urea as N-sources.

[0062] It is further demonstrated that the amyloglucosidase gene can be deleted from the genome of Aspergillus. A replacement vector is constructed containing a part of the glaA promoter, a synthetic DNA sequence containing stop codons in all three reading frames, the amdS gene from A. nidulans under the control of the A.nidulans glyceraldehyde-3-phosphate dehydrogenase promoter and wherein the amdS gene is flanked by 3' glaA non-coding sequences. After transformation of A.niger the vector is integrated by double crossing-over thereby effectively replacing the amyloglucosidase gene. After selection for amdS activity the transformed strains are plated on fluoracetamide and urea. Selection resulted in strains wherein the amdS gene was deleted.

[0063] This example is an illustration of the possibility of using the amdS gene for deletion of a desired gene from the genome of an Aspergillus strain. Other genes can be eliminated or modified in a similar manner.

[0064] In a further example it is demonstrated that a gene can be inserted marker free at a predetermined site in the genome. An integration vector is constructed containing the A.niger glaA locus and the amdS gene flanked by two 3' glaA non-coding repeats.

[0065] The construct is shown to integrate at the amyloglucosidase locus. After selection on fluoracetamide the amdS gene is deleted. In this way a gene copy is integrated at a specific locus without leaving marker DNA.

[0066] It is evident from the above that the procedures described herein enable one of skill in the art to integrate or delete desired genes at predetermined loci without leaving selection marker DNA behind.

[0067] This method can be employed for gene amplification and gene replacement.

[0068] An especially important application would be the integration of desired genes. Followed by classical strain improvement whereafter the genes that may be adversely affected by the classical strain improvement techniques are replaced with fresh unaffected copies of the gene of interest without loss of expression level.

[0069] The system as described for Aspergillus above is expected to give the same results when other filamentous fungal strains are employed, which are known to be incapable of growth on acetamide as the sole N-source. The use

of the amdS gene as a selection marker has been described for among others Penicillium and Trichoderma. Moreover, the amdS gene can even be used in filamentous fungi which are capable of using acetamide as sole N-source albeit poorly. In this case the background of poorly growing untransformed cells can be repressed by the inclusion of CsCl in the selection media (Tilburn, J. et al. (1983) Gene, 26, 205-221). Hence the system is expected to be applicable to filamentous fungi in general.

[0070] The advantages of the system of the present invention are manifold. The most striking advantages are given below:

- It is demonstrated that the amdS system is universally applicable in filamentous fungi requiring only that the host in question cannot or only poorly grow on acetamide as sole cor N-source but can utilize either acetate or ammonia as sole C- or N-source, respectively.
- The amdS system represents the only bi-directional and dominant selection system. This feature is extremely convenient for use in poly- or aneuploid strains which often is the case with natural isolates and/or industrial filamentous fungal strains.
- After classical strain improvement any mutated copies of the desired gene can be easily replaced by unmutated copies by gene replacement due to the fact that the desired genes have been integrated at well-defined loci. The genes are thus replaced with unmutated genes without affecting the expression level.
- Due to the ability to introduce multiple integrations at well-defined and therefore non-random loci one can be assured that no undesirable traits arise in the filamentous fungal strain upon gene amplification.
- The growing concern about the release of various selection markers in the environment is overcome by the presented system. No selection marker gene or other unnecessary or undesired DNA sequences need to be present in the filamentous fungal production strains after introduction of the desired genes or other genetic modifications.

Experimental

General molecular cloning techniques

[0071] In the examples described herein, standard molecular cloning techniques such as isolation and purification of nucleic acids, electrophoresis of nucleic acids, enzymatic modification, cleavage and/or amplification of nucleic acids, transformation of E.coli, etc., were performed as described in the literature (Sambrook et al. (1989) "Molecular Cloning: a laboratory manual", Cold Spring Harbour Laboratories, Cold Spring Harbour, New York; Innis et al. (eds.) (1990) "PCR protocols, a guide to methods and applications" Academic Press, San Diego). Synthesis of oligo-deoxynucleotides and DNA sequence analysis were performed on an Applied Biosystems 380B DNA synthesizer and 373A DNA sequencer, respectively, according to the user manuals supplied by the manufacturer.

Transformation of A.niger

[0072] Transformation of A.niger was performed according to the method described by Tilburn, J. et.al. (1983) Gene 26, 205-221 and Kelly, J. & Hynes, M. (1985) EMBO J., 4, 475-479 with the following modifications:

- spores were grown for 16 hours at 30°C in a rotary shaker at 300 rpm in Aspergillus minimal medium. Aspergillus minimal medium consists of the following components: Per liter: 6 g $NaNO_3$; 0.52 g KCl; 1.52 g KH_2PO_4; 1.12 ml 4M KOH; 0.52 g $MgSO_4.7H_2O$; 10 g glucose; 1 g casamino acids; 22 mg $ZnSO_4.7H_2O$; 11 mg H_3BO_3; 5 mg $FeSO_4.7H_2O$; 1.7 mg $CoCl_2.6H_2O$; 1.6 mg $CuSO_4.5H_2O$; 5 mg $MnCl_2.4H_2O$; 1.5 mg $Na_2MoO_4.2H_2O$; 50 mg EDTA; 2 mg riboflavin; 2 mg thiamine.HCl; 2 mg nicotinamide; 1 mg pyridoxine.HCl; 0.2 mg panthotenic acid; 4 µg biotin; 10 ml Penicillin (5000 IU/ml)/Streptomycin (5000 UG/ml) solution (Gibco).
- only Novozym 234 (Novo Industri), and no helicase, was used for formation of protoplasts;
- after protoplast formation (60-90 minutes), KC buffer (0.8 M KCl, 9.5 mM citric acid, pH6.2) was added to a volume of 45 ml. and the protoplast suspension was centrifuged at 2500 g at 4°C for 10 minutes in a swinging-bucket rotor. The protoplasts were resuspended in 20 ml. KC buffer. Then, 25 ml of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl pH7.5, 50 mM $CaCl_2$) was added and subsequently the protoplast suspension was centrifuged at 2500 g at 4°C for 10 minutes in a swinging-bucket rotor, washed in STC-buffer and resuspended in STC-buffer at a concentration of 10^8 protoplasts/ml;
- to 200 µl of the protoplast suspension the DNA fragment, in a volume of 10 µl in TE buffer (10 mM Tris-HCl pH7.5, 0.1 mM EDTA), was added and subsequently 100 µl of a PEG solution (20% PEG 4000 (Merck), 0.8 M sorbitol, 10 mM Tris-HCl pH7.5, 50 mM $CaCl_2$);
- after incubation of the DNA-protoplast suspension at room temperature for 10 minutes, 1.5 ml PEG solution (60% PEG 4000 (Merck), 10 mM Tris-HCl pH7.5, 50 mM $CaCl_2$) was added slowly, with repeated mixing of the tubes.

After incubation at room temperature for 20 minutes, the suspensions were diluted with 5 ml STC buffer, mixed by inversion and centrifuged at 2000 g at room temperature for 10 minutes. The protoplasts were resuspended gently in 1 ml 1.2 M sorbitol and plated onto selective regeneration medium consisting of Aspergillus minimal medium without riboflavin, thiamine.HCl, nicotinamide, pyridoxine.HCl, panthotenic acid, biotin, casamino acids and glucose but with 10 mM acetamide as the sole nitrogen source, 1 M sucrose, solidified with 2% bacteriological agar #1 (Oxoid, England). Following growth for 6-10 days at 30°C, the plates were replica plated onto selective acetamide plates consisting of Asperqillus selective regeneration medium with 2% glucose instead of sucrose and 1.5% agarose instead of agar. Single transformants were isolated after 5-10 days of growth at 30°C.

Transformation of A. oryzae

[0073] Transformation of A. oryzae was performed according to the method described by Christensen, T. et al. in European Patent Application 0 238 023 A2.

Transformation of T. reesei

[0074] Transformation of T. reesei was performed according to the method described by Penttilla M., Knowles, J. (1987) Gene 61 155-164.

Transformation of P. chrysogenum

[0075] The Ca-PEG mediated protoplast transformation procedure is used. Preparation of protoplasts and transformation of P.chrysogenum was performed according to the method described by Gouka et al., Journal of Biotechnology 20(1991), 189-200 with the following modifications:

- After transformation, the protoplasts were plated onto selective regeneration medium plates consisting of Aspergillus minimal medium, osmotically stabilized with 1.2 M sucrose, containing 0.1% acetamide as sole nitrogen source and solidified with 1.5% bacteriological agar #1 (Oxoid, England).
- After 5-8 days of incubation at 25°C transformants appeared.

Isolation of DNA from Aspergillus, Trichoderma, Penicillium

[0076] The isolation of DNA from Aspergillus and Trichoderma was performed according to the procedure as described by Yelton, et al. (1984), Proc. Natl. Acad. Sci. 81, 1470-1474.

[0077] The isolation of DNA from Penicillium was performed according to the procedure described by Kolar et al., Gene 62 (1988), 127-134.

Removal of the amdS selection marker

[0078] The amdS marker in most examples relating to Aspergillus, Trichoderma and Penicillium is cloned between repeats consisting of a part of the 3' non-coding region of amyloglucosidase gene. Removal of the amdS selection marker is achieved either by internal recombination between the 3' glaA non-coding repeats that flank the amdS selection marker or by homologous recombination between the repeats that are created by integration via a single cross-over event. Selection of cells that have lost the amdS selection marker is achieved by growth on plates containing fluoracetamide. Cells harbouring the amdS gene metabolize fluoracetamide to ammonium and fluoracetate which is toxic to the cell. Consequently, only cells that have lost the amdS gene are able to grow on plates containing fluoracetamide.

[0079] In case of removal of the amdS marker from Aspergillus transformants, spores from these transformants were plated onto selective regeneration medium (described above) containing 32 mM fluoracetamide and 5 mM ureum instead of 10 mM acetamide, 1.1% glucose instead of 1M sucrose and 1.1% instead of 2% bacteriological agar #1 (Oxoid, England). After 7-10 days of growth at 35°C single colonies were harvested and plated onto 0.4% potato dextrose agar (Oxoid, England). In case of removal of the amdS marker from Trichoderma transformants, spores of these transformants were plated onto non selective minimal medium plates (per liter: 20 g. glucose, 5 g. $(NH_4)_2SO_4$, 15 g. KH_2PO_4, 0.6 g. $MgSO_4$, 0.6 g. $CaCl_2$, 0.005 g. $FeSO_4.7H_2O$, 0.0016 g. $MnSO_4.H_2O$, 0.0014 g. $ZnSO_4.7H_2O$, 0.002 g. $CoCl_2$; pH5.5) supplemented with 10 mM fluoracetamide. After 5-10 days at 30°C, colonies were harvested and plated onto 0.4% potato dextrose agar (Oxoid, England).

[0080] In case of removal of the amdS marker from Penicillium transformants, spores from these transformants were plated on selective medium plates consisting of Aspergillus minimal medium with 10 mM fluor-acetamide and 5%

glucose, solidified with 1.5% bacteriological agar #1 (Oxoid, England). After 5-10 days of growth at 25°C resistant colonies appeared.

Determination of glucoamylase production by A.niger transformants

[0081] Of recombinant and control A.niger strains spores were collected by plating spores or mycelia onto PDA-plates (Potato Dextrose Agar, Oxoid), prepared according to the supplier's instructions. After growth for 3-7 days at 30°C spores were collected after adding 0,01% Triton X-100 to the plates. After washing with sterile water approximately 10^7 spores of selected transformants and control strains were inoculated into shake flasks, containing 20 ml of liquid preculture medium containing per litre: 30 g maltose.H_2O; 5 g yeast extract; 10 g hydrolysed casein; 1 g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; 3 g Tween 80; 10 ml Penicillin (5000 IU/ml)/Streptomycin (5000 UG/ml); pH 5.5. These cultures were grown at 34°C for 20-24 hours. 5-10 ml of this culture was inoculated into 100 ml of fermentation medium containing per litre: 70 g maltodextrines; 25 g hydrolysed casein; 12.5 g yeast extract; 1 g KH_2PO_4; 2 g K_2SO_4; 0.5 g $MgSO_4.7H_2O$; 0.03 g $ZnCl_2$; 0.02 g $CaCl_2$; 0.01 g $MnSO_4.4H_2O$; 0.3 g $FeSO_4.7H_2O$; 10 ml penicillin (5000 IU/ml)/Streptomycin (5000 UG/ml); adjusted to pH 5.6 with 4 N H_2SO_4. These cultures were grown at 34°C for 5-10 days. Samples were taken for the analysis of the glucoamylase production at different time points during fermentation. Fermentation broth samples were centrifuged (10 minutes, 10.000xg) and supernatants collected.

[0082] The glucoamylase activity was determined by incubating 10 µl of a six times diluted sample of the culture supernatant in 0.032 M NaAC/HAC pH4.05 with 115 µl of 0.2% (w/v) p-Nitrophenyl α-D-glucopyranoside (Sigma) in 0.032 M NaAc/HAc pH 4.05. After a 30 min incubation at room temperature, 50 µl of 0.3 M Na_2CO_3 was added and the absorption at a wavelength of 405 nm was measured. The A_{405na} is a measure for the AG production.

Example 1

Marker gene free deletion of an A.niger gene by using the amdS gene

[0083] In this example a genomic target gene in A.niger will be replaced by transforming A.niger with a replacement vector which integrates into the A.niger genome via a double cross-over homologous recombination. The replacement vector comprises a DNA region homologous to the target locus interrupted by a selectable marker gene flanked by DNA repeats.

[0084] In this example plasmid pGBDEL4L is used to delete the glaA coding region and a (proximal) part of the glaA promoter region. This vector comprises a part of the A.niger glaA genomic locus, wherein the glaA coding sequences as well as a part of the glaA promoter sequences are replaced by the A.nidulans amdS gene under the control of A. nidulans gpdA promoter as selection marker flanked by 3'-untranslated glaA sequences as direct repeats. Transformation of A.niger with this vector directs replacement of the glaA gene by the amdS marker gene. By performing the fluoracetamide counter-selection on these transformants as described in the experimental procedures, the amdS marker gene will be deleted properly by an internal recombination event between the 3'glaA DNA repeats, resulting in a marker gene free ΔglaA recombinant strain, possessing finally no foreign DNA sequences at all (for a schematic view, see Figure 2).

Short description of the glaA gene replacement vector pGBDEL4L

[0085] The gene replacement vector pGBDEL4L contains 5'-part of the A.niger amyloglucosidase (glaA) promoter region, a synthetic DNA sequence of 16 bp providing stopcodons in all three reading frames, the A.nidulans acetamidase (amdS) gene under control of the A.nidulans glyceraldehyde-3-phosphate dehydrogenase (gpdA) promoter, flanked at both sides by 3' glaA non-coding sequences.

Construction pathway of pGBDEL4L

[0086] In order to obtain the final deletion vector pGBDEL4L several subclones of the glaA locus were derived first. A schematic view is presented in figure 3. The glaA locus of A.niger was molecular cloned and described previously (EP 0 463 706 A1). Plasmid pAB6-1 contains the entire glaA locus from A.niger on a 15.5 kb HindIII fragment cloned in the HindIII site of pUC19 (Yanisch-Perron et al., Gene 33 (1985) 103-119, and is obtainable from e.g. Boehringer Mannheim, Germany). pAB6-1 was digested with EcoRI and the 1.8 kb EcoRI DNA fragment just upstream of the glaA gene was isolated by agarose gel electrophoresis and ligated into pUC19 digested with EcoRI and subsequently transferred to E. coli and molecular cloned. The resulting plasmid was designated pAB6-3 (Figure 3A). To construct plasmid pAB6-4, which is another subclone of pAB6-1, pAB6-1 was digested with HindIII and BglII. The 4.6 kb sized DNA fragment comprising the glaA promoter and a part of the glaA coding sequence was isolated by agarose gel electro-

phoresis and ligated into pUC19 which was digested prior with HindIII and BamHI (Figure 3B). As a result the BamHI as well as the BglII sites in pAB6-4 were destroyed appropriately by this cloning procedure.

[0087] Subsequently, after digesting plasmid pAB6-4 with HindIII and EcoRI and filling in the 5' sticky ends using E. coli DNA polymerase, the 1.8 kb glaA promoter DNA fragment was isolated by agarose gel electrophoresis, ligated into pAB6-3 which was partially digested with EcoRI and treated with E. coli DNA polymerase to generate blunt ends, the ligation mixture was transferred to E. coli for molecular cloning. The derived plasmid (designated pAB6-31) contains a 3.6 kb glaA promoter fragment with a destroyed EcoRI site in the middle, but still possessing the EcoRI site (now unique in this DNA fragment) just upstream of the glaA ATG initiation site (Figure 4).

[0088] The A.nidulans amdS gene used herein is located on an approximately 4 kb sized EcoRI-KpnI fragment in plasmid pGW325 (Wernars et al., thesis (1986) Agricultural University, Wageningen, The Netherlands). This EcoRI-KpnI DNA fragment containing the amdS gene, flanked by its own regulatory sequences, was molecular cloned into the appropriate sites of pUC19 as described by Verdoes et al. (Transgenic Res. 2 pp 84-92, 1993) resulting in pAN4-1. pAN4-1 was digested with EcoRI and KpnI, the 4 kb sized DNA fragment containing the amdS gene was isolated by agarose gel electrophoresis, ligated into pAB6-31 digested with EcoRI and KpnI and the ligation mixture was transferred to E. coli for molecular cloning. The obtained plasmid was designated pAB6S (Figure 5) and contains a 3.8 kb glaA promoter DNA fragment and the 4 kb amdS fragment.

[0089] Plasmid pAB6S was first partially digested with SalI, and ligated to the synthetic derived oligonucleotide TN0001 (SEQ ID NO: 3) having the following sequence:

TN0001 (SEQ ID NO: 3): 5' TCGATTAACTAGTTAA 3'

and secondly digested with EcoRI. The DNA fragment comprising the pUC19, the glaA promoter and the amdS gene sequences was purified and isolated by agarose gel electrophoresis. From plasmid pAB6-1, digested with SalI, the 2.2 kb 3' flanking glaA DNA fragment was isolated as well by agarose gel electrophoresis and ligated to the above mentioned synthetic oligonucleotide, treated with T4 polynucleotide kinase, subsequently digested with EcoRI and ligated to the above mentioned DNA fragment isolated of pAB6S. The DNA ligation mixture was transferred to E. coli and molecular cloned. The derived plasmid was designated pGBDEL1 and is shown in Figure 6. By this procedure simultaneously the SalI restriction site was destroyed and stopcodons in all reading frames were introduced.

[0090] To obtain an approximately 1 kb large DNA fragment, containing 3' glaA non-coding DNA sequences positioned just downstream the stop codon of the glaA gene and flanked by suitable restriction sites, a PCR amplification was performed. In this PCR amplification, the plasmid pAB6-1 was used as template and as primers two synthetical derived oligonucleotides:

Oligo AB2154 (SEQ ID NO: 4):
5'AACCATAGGGTCGACTAGACAATCAATCCATTTCG 3'
(a 3'glaA non-coding sequence just downstream of the stopcodon) and

Oligo AB2155 (SEQ ID NO: 5):
5'GCTATTCGAAAGCTTATTCATCCGGAGATCCTGAT 3'
(a 3'glaA non-coding sequence around the EcoRI site approx. 1 kb downstream of the stopcodon).

[0091] The PCR was performed as described by Saiki et al. (Science 239, 487-491, 1988) and according to the supplier of TAQ-polymerase (Cetus). Twenty five amplification cycles (each 2 minutes at 55 °C; 3 minutes at 72 °C and 2 minutes at 94°C) were performed in a DNA-amplifier (Perkin-Elmer/Cetus). The 1 kb amplified DNA fragment was digested with HindIII and SalI, purified by agarose gel electrophoresis, ethanol precipitated and subsequently cloned into the HindIII and SalI restriction sites of pGBDEL1. The thus obtained plasmid was designated pGBDEL2

(Figure 7A,B).

[0092] To obtain the final glaA gene replacement vector pGBDEL4L, the amdS promoter region in pGBDEL2 was exchanged by the stronger A.nidulans gpdA promoter. Fusion of the gpdA promoter sequence to the coding sequence of the amdS gene was performed by the Polymerase Chain Reaction (PCR) method. For this PCR fusion two different templates were used: plasmid pAN7-1 (Punt et al., Gene 56, 117-124, 1987) containing the E.coli hph gene under control of the A.nidulans gpdA promoter and the A.nidulans trpC terminator and plasmid pAN4-1, containing the A. nidulans amdS gene under control of its own regulatory sequences. As primers four synthetic oligonucleotides were used, possessing the following sequences:

Oligo AB 2977 (SEQ ID NO: 6):
5' TATCAGGAATTCGAGCTCTGTACAGTGACC 3'

(a 5' gpdA promoter specific oligo nucleotide, positioned at approximately 880 bp upstream of the ATG startcodon of the E. coli hph gene)

Oligo AB2992 (SEQ ID NO: 7):
5' GCTTGAGCAGACATCACCATGCCTCAATCCTGGGAA 3'

Oligo AB2993 (SEQ ID NO: 8):
5' TTCCCAGGATTGAGGCATGGTGATGTCTGCTCAAGC 3'

(both sequences are complementary to each other and contain 18 bp of the 3' end of the gpdA promoter and 18 bp of the 5' part of the amdS coding region)

Oligo AB2994 (SEQ ID NO: 9):
5' CTGATAGAATTCAGATCTGCAGCGGAGGCCTCTGTG 3'

(an amdS specific sequence around the BglII site approximately 175 bp downstream of the ATG initiation codon)

[0093] To fuse the 880 bp gpdA promoter region to the amdS coding sequence two separate PCR's were carried out: the first amplification with pAN7-1 as template and the oligo nucleotides AB 2977 (SEQ ID NO: 6) and AB2993 (SEQ ID NO: 8) as primers to amplify the 880 bp DNA fragment comprising the gpdA promoter flanked at the 3' border by 18 nucleotides complementary to the 5' end of the amdS gene, and the second PCR reaction with pAN4-1 as template and the oligo nucleotides AB2992 (SEQ ID NO: 7) and AB2994 (SEQ ID NO: 9) as primers to amplify a 200 bp sized DNA fragment comprising the 5' part of the amdS gene flanked at the 5' border by 18 nucleotides complementary to the 3' end of the gpdA promoter. A schematic view of these amplifications is presented in Figure 8A. The two fragments generated were subsequently purified by agarose gel electrophoresis, ethanol precipitated and used as templates in a third PCR reaction with oligo nucleotides AB 2977 (SEQ ID NO: 6) and AB2994 (SEQ ID NO: 9) as primers. The resulting DNA fragment was digested with EcoRI, purified by agarose gel electrophoresis and ethanol precipitation, and cloned into the EcoRI site of pTZ18R (United States Biochemicals). The resulting plasmid was designated pGBGLA24 (Figure 8B).

[0094] To exchange the amdS promoter sequence in pGBDEL2 by the gpdA promoter sequence, the approximately 1 kb sized EcoRI/BglII DNA fragment of pGBGLA24 was isolated by agarose gel electrophoresis after digestion with the appropriate restriction enzymes and ligated into the EcoRI and BglII sites of pGBDEL2. The resulting glaA gene replacement vector was designated pGBDEL4L (Figure 9).

Deletion of glaA promoter and coding sequences in A.niger

[0095] Prior to transformation of A.niger with pGBDEL4L, the E.coli sequences were removed by HindIII and XhoI digestion and agarose gel electrophoresis. The A.niger strain CBS 513.88 (deposited October 10, 1988) was trans-

formed with either 2.5, 5 or 10 µg DNA fragment by procedures as described in experimental procedures using acetamide as sole N-source in selective plates. Single A.niger transformants were purified several times onto selective acetamide containing minimal plates. Spores of individual transformants were collected by growing for about 5 days at 30°C on 0.4% potato-dextrose (Oxoid, England) agar plates. Southern analyses were performed to verify the presence of the truncated glaA locus. High molecular weight DNA of several transformants was isolated, digested with BamHI and KpnI and subsequently fractionated by electrophoresis on a 0.7% agarose gel. After transfer to nitrocellulose filters, hybridization was performed according to standard procedures using two ^{32}P-labelled probes: a XhoI/SalI glaA promoter fragment isolated from plasmid pAB6-4 (described above, Figure 3A) and a probe recognizing endogenous xylanase sequences (European Patent Application. 0 463 706 A). The results of only 4 transformants (#19, #23, #24, #41) and the control strain A.niger CBS 531.88 are shown as examples in Figure 10A. For a better understanding of this autoradiograph, a schematic presentation is presented in Figure 11 showing the size of the hybridizing fragments in intact and truncated glaA loci.

[0096] Characteristic for the intact glaA locus is a 3.5 kb hybridizing fragment in a BamHI digest and a 4.5 kb hybridizing fragment in a KpnI digest (see figure 11A). In a truncated glaA locus, the 3.5 kb BamHI hybridizing fragment and the 4.5 kb KpnI hybridizing fragment are absent and replaced by a 5.5 kb BamHI hybridizing fragment and a 6.3 kb KpnI hybridizing fragment. In this example; as can be seen in Figure 10A, transformant #19 shows the expected pattern of a truncated glaA locus (Figure 11B). This transformant was designated GBA-102.

[0097] No replacement of the glaA gene had occurred in the other transformants. The poorly hybridizing bands: 4, 8 and 15 kb in the KpnI digest and 7 and 12 kb in the BamHI digest, refer to the xylanase sequences as internal control.

Removal of the amdS gene from A.niger GBA-102 by counter-selection on fluoracetamide containing plates.

[0098] The amdS gene in the transformant A.niger GBA-102 was removed again as described in the Experimental section. The removal of the amdS selection marker gene in only 2 surviving recombinant strains was verified by Southern analysis of the chromosomal DNA. High molecular weight DNA was isolated, digested with BamHI and KpnI and subsequently separated by electrophoresis on a 0.7% agarose gel. Following transfer to nitrocellulose hybridization was performed according to standard procedures using the probes described in the previous section. A schematic presentation of the hybridizing fragments is shown in Figure 11c. The results of the Southern analyses are presented in Figure 10B. The presence of a 5.2 kb hybridizing BamHI fragment and a 3.4 kb hybridizing KpnI fragment, with the concomitant loss of the 5.5 kb BamHI and the 6.3 kb hybridizing KpnI fragments is specific for the absence of the amdS selection marker. The weaker hybridizing 7 and 12 kb fragments in a BamHI digest and the 4, 8 and 15 kb KpnI fragments again refer to the endogenous xylanase locus. Both strains show the expected pattern. In these recombinant strains, which were designated GBA-107 and GBA-108, the preferred glaA sequences are removed correctly and that possess finally no selection marker gene at all. Both strains can be reused again to delete or insert other genes or DNA elements by using the same type of vector.

Example 2

Marker gene free introduction of the glaA gene targeted at the 3'glaA non-coding region of the truncated glaA locus in A.niger GBA-107

[0099] In this example the introduction of a gene into the genome of A.niger is described by using approximately the same approach and procedures as described in the previous example. Besides the desired gene or DNA element the vector contains DNA sequences homologous to the host genome to target the vector at a predefined genomic locus of the host, by a single cross-over event. This type of vector comprises a selection marker gene flanked by DNA repeats as well. The selection marker gene in transformants derived with this vector can be removed properly again by applying the counter-selection procedure. As an example the introduction of a glaA gene copy is described which becomes integrated at the truncated glaA locus in the recombinant ΔglaA A.niger GBA-107 strain derived in Example I (for a schematic drawing see Figure 12)

Description of the glaA integration vector: pGBGLA30

[0100] The integration vector pGBGLA30 consists of the A.niger amyloglucosidase (glaA) gene under control of the native promoter and the A.nidulans amdS gene under control of the A.nidulans gpdA promoter flanked by 3'glaA non-coding sequences to direct integration at the 3' glaA non-coding region and to remove the amdS selection marker gene via the counter-selection.

Construction of the integration vector

[0101] A 1.8 kb XhoI/EcoRI glaA promoter fragment from pAB6-1 (Figure 13) was subcloned into the SmaI and EcoRI sites of pTZ19R (United States Biochemicals). The protruding 5' end of the XhoI site of the glaA promoter fragment was filled in using the Klenow fragment of E.coli DNA polymerase I prior to cloning in pTZ19R. The SmaI site is destroyed and the XhoI site is restored by this cloning procedure. The thus obtained plasmid was designated pGBGLA5 (Figure 13).

[0102] To introduce appropriate restriction sites (AatII, SnaBI, AsnI and NotI) and to destroy the XhoI site in the glaA promoter, the synthetic fragment consisting of the two oligonucleotides AB3657 (SEQ ID NO: 10) and AB3658 (SEQ ID NO: 11):

```
5' AGCTTGACGTCTACGTATTAATGCGGCCGCT      3'  AB3657
   ||||||||||||||||||||||||||||||
3'     ACTGCAGATGCATAATTACGCCGGCGAAGCT  5'  AB3658
```

was inserted into the HindIII and XhoI sites of pGBGLA5. The thus obtained plasmid was designated pGBGLA26 (Figure 14).

[0103] Next, the 3.4 kb EcoRI fragment from pAB6-1 containing the remaining 3' part of the glaA promoter, the glaA coding sequence and part of the 3' glaA non-coding sequence, was cloned into the EcoRI site of pGBGLA26. This new plasmid was designated pGBGLA27 (Figure 15). This plasmid was partially digested with EcoRI and the synthetic fragment consisting of the oligonucleotides AB3779 (SEQ ID NO: 12) and AB3780 (SEQ ID NO: 13):

```
5' AATTGGGGCCCATTAACTCGAGC      3'  AB3779
   ||||||||||||||||||||||
3'     CCCCGGGTAATTGAGCTCGTTAA  5'  AB3780
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was inserted into the EcoRI site at the end of the 3' glaA non-coding sequence from the glaA gene. By this cloning step, the EcoRI site was destroyed and an ApaI and XhoI restriction site were introduced. The resultant plasmid was designated pGBGLA42 (Figure 16).

[0104] Amplification of the 2.2 kb 3' glaA non-coding sequences and concomitant adjustment of appropriate restriction sites was performed by the Polymerase Chain Reaction (PCR) method.

[0105] In these PCR reactions, plasmid pAB6-1 containing the entire glaA locus was used as template and as primers four synthetic oligo nucleotides were designed possessing the following sequence:

Oligo AB3448 (SEQ ID NO: 14):

5' GTGCGAGGTACCACAATCAATCCATTTCGC 3'

(a 3' glaA non-coding specific sequence just downstream the stopcodon of the glaA gene)

Oligo AB3449 (SEQ ID NO: 15):

5' ATGGTTCAAGAACTCGGTAGCCTTTTCCTTGATTCT 3'

(a 3' glaA non-coding specific sequence around the KpnI site approx. 1 kb downstream of the stop codon)

Oligo AB3450 (SEQ ID NO: 16):

5' AGAATCAAGGAAAAGGCTACCGAGTTCTTGAACCAT 3'

(a 3' glaA non-coding specific sequence around the KpnI site approx. 1 kb downstream of the stop codon)

Oligo AB3520 (SEQ ID NO: 17):

5'ATCAATCAGAAGCTTTCTCTCGAGACGGGCATCGGAGTCCCG 3'

(a 3' glaA non-coding specific sequence approx. 2.2 kb downstream of the stopcodon)

[0106] To destroy the KpnI site approximately 1 kb downstream of the stop codon from the glaA gene and to alter the SalI site approximately 2.2 kb downstream the stop codon from the glaA gene into a XhoI site two separate polymerase chain reactions were performed: the first reaction with oligonucleotides AB3448 (SEQ ID NO: 14) and AB3449 (SEQ ID NO: 15) as primers to amplify an approximately 1 kb DNA fragment just downstream the stopcodon of the glaA gene, and the second reaction with oligonucleotides AB3450 (SEQ ID NO: 16) and AB3520 (SEQ ID NO: 17) as primers to amplify an approximately 1.2 kb DNA fragment just downstream the KpnI site in the 3' glaA non-coding region both with pAB6-1 as template. A schematic view of these amplifications is presented in Figure 17A. The PCR was performed as described in example I. Twenty-five amplification cycles (each 1 minute at 55°C; 1.5 minutes at 72°C and 1 minute at 94°C) were carried out.

[0107] The two generated PCR DNA fragments were purified by agarose gel electrophoresis and ethanol precipitation and subsequently used as template in the third PCR with oligonucleotides AB3448 (SEQ ID NO: 14) and AB3520 (SEQ ID NO: 17) as primers to generate the fusion fragment. Twenty-five amplification cycles (each: 2 minutes at 55°C; 3 minutes at 72°C; 2 minutes at 94°C) were carried out in a DNA-amplifier (Perkin-Elmer/Cetus). The amplified DNA fragment was purified by agarose gel electrophoresis and ethanol precipitation and subsequently subcloned in the SmaI site of pTZ18R. The obtained plasmid was designated pGBGLA17 (Figure 17B).

[0108] To fuse this adjusted 3' glaA non-coding region to the amdS gene, a part of the amdS gene was subcloned from pGBDEL4L into pSP73 (Promega). For this construction, pGBDEL4L was digested with BglII and HindIII, the 3.4 kb amdS/3'glaA non-coding fragment was isolated by agarose gel electrophoresis and subcloned into the appropriate sites of pSP73 (Promega). The resulting plasmid was designated pGBGLA21 (Figure 18).

[0109] The approximately 1 kb sized 3' glaA non-coding region in this plasmid was exchanged by the 2.2 kb 3' glaA non-coding region of pGBGLA17. pGBGLA17 and pGBGLA21 were digested with KpnI and HindIII. The 2.2 kb 3' glaA non-coding region DNA fragment from pGBGLA17 and the 4.9 kb DNA fragment of pGBGLA21 were isolated by agarose gel electrophoresis, ligated and subsequently molecular cloned by transferring the ligation mixture to E. coli. The thus derived plasmid was designated pGBGLA22 (Figure 19).

[0110] The amdS gene with the extended 3'glaA non-coding region was completed with the gpdA promoter and fused to the remaining part of the amdS gene. pGBGLA22 was digested with BglII and HindIII, the 4.4 kb amdS/3'glaA non-coding region DNA fragment isolated by agarose gel electrophoresis, subsequently ligated with plasmid pGBGLA24 digested with BglII and HindIII and transferred to E. coli. The thus derived plasmid was designated pGBGLA25 (Figure 20).

[0111] pGBGLA25 was partially digested with EcoRI and in the EcoRI site of the gpdA promoter the synthetic fragment consisting of the two oligonucleotides AB3781 (SEQ ID NO: 18) and AB3782 (SEQ ID NO: 19):

```
5'  AATTGGGGCCCAGCGTCC        3'   AB3781
    ||||||||||||||||
3'      CCCCGGGTCGCAGGTTAA    5'   AB3782
```

was inserted. This new plasmid was designated pGBGLA43 (Figure 21). Due to this cloning step, the EcoRI restriction site just in front of the gpdA promoter was destroyed by the introduction of an ApaI restriction site.

[0112] The plasmid pGBGLA43 was digested with ApaI and XhoI, and the 5.3 kb DNA fragment comprising the gpdA promoter/amdS gene/3'glaA non-coding region was isolated by agarose gel electrophoresis, subsequently ligated with pGBGLA42 digested with ApaI and XhoI, and transferred to E.coli. The derived plasmid was designated pGBGLA28 (Figure 22).

[0113] Prior to cloning, the 3'glaA non-coding region DNA fragment (positioned at approximately 2.2 kb downstream the stop codon of the glaA gene, designated 3"glaA non-coding region), was amplified and provided with suitable restriction sites using the PCR method.

[0114] For this PCR reaction, the plasmid pAB6-1 was used as template and as primers two synthetic oligonucleotides were designed possessing the following sequence:

Oligo AB3746 (SEQ ID NO: 20):

5' TGACCAATAAAGCTTCTCGAGTAGCAAGAAGACCCAGTCAATC 3'

(a partly 3"glaA non-coding specific sequence around the SalI site positioned at about 2.2 kb downstream the stop codon of the glaA gene)

Oligo AB3747 (SEQ ID NO: 21):

5'CTACAAACGGCCACGCTGGAGATCCGCCGGCGTTCGAAATAACCAGT3'

(a partly 3"glaA non-coding specific sequence around the XhoI site located at about 4.4 kb downstream the stop codon of the glaA gene)

[0115] Twenty-five amplification cycles (each: 1 minute 55°C; 1.5 minutes 72°C; 1 minute 94°C) were carried out in a DNA-amplifier (Perkin-Elmer/Cetus). A schematic representation of this amplification is shown in figure 23A. The thus obtained DNA fragment was digested with HindIII, purified by agarose gel electrophoresis and ethanol precipitation and subcloned in both orientations into the HindIII site of pTZ19R. The resulting plasmids were designated pGBGLA29A and pGBGLA29B (Figure 23).

[0116] The final step comprises the insertion of the 3"glaA non-coding sequence from pGBGLA29A into the plasmid pGBGLA28. To achieve this, pGBGLA29A was digested with HindIII and NotI. The 2.2 kb sized 3'glaA non-coding region fragment was isolated by agarose gel electrophoresis, subsequently ligated to pGBGLA28 digested with HindIII and NotI and transferred to E. coli. The derived integration vector was designated pGBGLA30 (Figure 24).

Transformation of A.niger GBA-107 with the integration vector pGBGLA30

[0117] Prior to transformation, E.coli sequences were removed from the integration vector pGBGLA30 by XhoI digestion and agarose gel electrophoresis. The A.niger strain GBA-107 was transformed with either 5 or 10 µg DNA fragment by procedures as described in the experimental section. Single A.niger transformants were purified several times on selective acetamide containing plates. Spores of individual transformants were collected following growth for about 5 days at 30°C on 0.4% potato dextrose agar (Oxoid, England) plates. Southern analyses were performed to verify whether integration into the 3' glaA non coding region of the endogenous truncated glaA locus had occurred. High molecular weight DNA of several transformants was isolated, digested with either KpnI, or BglII and subsequently fractionated by electrophoresis on a 0.7% agarose gel. After transfer to nitrocellulose filters, hybridization was performed according to standard procedures. As probe a ^{32}P-labelled approx. 0.7 kb XhoI/SalI glaA promoter fragment isolated from plasmid pAB6-4 (described in example 1) was used. The results of only 3 transformants (#107-5, #107-9 and #107-7) and the reference strain A.niger GBA107 and its ancestor A.niger CBS 531.88 are shown as example in Figure 25. For a better understanding of the autoradiograph, a schematic presentation is given in Figure 26A,B,C showing the sizes of the hybridizing fragments of the intact glaA locus, the truncated glaA locus and of the truncated glaA locus with a single pGBGLA30 copy integrated into the predefined 3' glaA non-coding region.

[0118] Characteristic for the intact glaA locus is a 4.5 kb hybridizing fragment in a KpnI digest and a 10 kb hybridizing fragment in a BglII digest. Characteristic for the truncated glaA locus of A.niger GBA-107 is a 3.4 kb hybridizing fragment in a KpnI digest and a 13 kb hybridizing fragment in a BglII digest. In case of integration of the pGBGLA30 vector into the 3' region of the truncated glaA locus, in a KpnI digest an additional 6.7 kb hybridizing fragment is expected besides the 3.4 kb hybridizing fragment and in a BglII digest the 13 kb hybridizing fragment is absent and replaced by a 14.5 kb hybridizing fragment. As can be seen in Figure 25, transformants #107-5 and #107-9 show the expected hybridization pattern of a single pGBGLA30 copy integrated into the predefined 3' non-coding region of the truncated glaA locus. The hybridization pattern of transformant #107-7 indicates integration of the pGBGLA30 copy elsewhere into the genome of A.niger GBA-107. The transformants with the correctly integrated pGBGLA30 copy were designated GBA-119 and GBA-122 and were used to remove subsequently the amdS selection marker gene properly.

Removal of the amdS selection marker gene from A.niger GBA-119 and GBA-122 by counter-selection on fluoracetamide containing plates.

[0119] The amdS selection marker gene in the transformants A.niger GBA-119 and GBA-122 was removed again as described in the experimental section. The removal of the amdS selection marker gene in several surviving recombinant strains was verified by Southern analysis of the chromosomal DNA. High molecular weight DNA was isolated,

digested either with KpnI or BglII and subsequently separated by electrophoresis on a 0.7% agarose gel. Following transfer to nitrocellulose, hybridization was performed according to standard procedures. As probe the ^{32}P labelled 2.2 kb HindIII/NotI 3"glaA non-coding fragment isolated from plasmid pGBGLA29A (described previously, Figure 24) was used.

[0120] A schematic presentation of the hybridizing fragments is shown in Figure 26. The results of only 3 surviving recombinant strains from A.niger GBA-119 (#AG5-5, #AG5-6 and #AG5-7) as well as 3 surviving recombinant strains from A.niger GBA-122 (#AG9-1, #AG9-2 and #AG9-4) and the reference strains A.niger CBS 531.88 and A.niger GBA-107 are shown in Figure 27A,B.

[0121] In strain A.niger CBS 531.88 a 6.9 kb hybridizing fragment is present in a KpnI digest and a 6.9 kb hybridizing fragment in a BglII digest. In the A.niger GBA-107 strain a 6.9 kb hybridizing fragment is present in a KpnI digest and a 13 kb hybridizing fragment in a BglII digest. In the A.niger strains GBA-119 and GBA-122 with a single pGBGLA30 copy integrated into the 3' glaA non-coding region an 8 kb and a 6.7 kb hybridizing band are present in a KpnI digest and a 14.5 kb and a 7.6 kb hybridizing band are present in a BglII digest.

[0122] Specific for correct removal of the amdS selection marker gene is the presence of a 6.7 kb and a 8.5 kb hybridizing fragment in a KpnI digest and concomitant loss of the 8 kb hybridizing fragment. In a BglII digest, a 14.5 kb and a 6.9 kb hybridizing fragment with concomitant loss of the 7.6 kb hybridizing fragment is specific for the absence of the amdS selection marker gene. As can be seen in Figure 27, strains #AG5-7, #AG5-5, #AG9-1 and #AG9-4 show the expected hybridizing pattern of the correctly removed amdS selection marker gene. These strains were designated GBA-120, GBA-121, GBA-123 and GBA-124 respectively. The hybridizing patterns of strains #AG5-6 and #AG9-2 indicate loss of the entire pGBGLA30 copy resulting in the parental A.niger GBA-107 strain with only a truncated glaA locus.

[0123] Strains A.niger GBA-120, GBA-121, GBA-123 and GBA-124 were tested in shake flask fermentations for the ability to produce glucoamylase. As reference strains A.niger CBS 531.88, GBA-107, GBA-119 and GBA-122 were tested. Shake flask fermentations and the glucoamylase assay were performed as described in the experimental section. In the strains GBA-119 till GBA-124 levels varying between 150-200 U/ml could be measured. These glucoamylase levels were to be expected and comparable to levels obtained with the parental untransformed wild-type strain A.niger CBS 531.88.

Example 3

Marker gene free introduction of the phytase gene targeted at the 3'glaA non-coding region of the truncated glaA locus in A.niger GBA-107

[0124] In this example describes the introduction of a gene into the genome of A.niger by using approximately the same approach and procedures as described in the previous example. The main difference is that the gene of interest and the selection marker gene are located on two separate vectors and that these vectors are co-transformed to A. niger. Besides the gene of interest or the marker gene, the vectors contain DNA sequences homologous to the host genome to target the vectors at a predefined genomic locus of the host, by a single cross-over event. By performing the fluoracetamide counter-selection on these (co)-transformants (as described in the experimental procedures), the amdS marker gene will be deleted properly by an internal recombination event between the DNA repeats that are created by integration via a single cross-over event.

Description of the vectors used for co-transformation

[0125] The vector with the gene of interest pGBGLA53 consists of the A.ficuum phytase gene under control of the A.niger glucoamylase (glaA) promoter flanked by 3'glaA non-coding sequences to direct integration at the 3'glaA non-coding region. The vector with the selection marker gene pGBGLA50 consists of the A.nidulans amdS gene under control of the A.nidulans gpdA promoter flanked by 3'glaA non-coding sequences to direct integration at the 3'glaA non-coding region.

Construction pathway of PGBGLA50

[0126] The construction of pGBGLA50 comprises one cloning step. Plasmid pGBGLA29A was digested with HindIII and the sticky ends were filled in using the Klenow fragment of E.coli DNA polymerase. Next, the 2.2 kb 3"glaA non-coding region fragment was isolated by agarose gel-electrophoresis, subsequently ligated into pGBGLA43 digested with ApaI and treated with T4 DNA polymerase to generate blunt ends, and transferred to E.coli. The derived plasmid with the 3"glaA non-coding region DNA fragment in the correct orientation was designated pGBGLA50 (Figure 28).

Construction pathway of pGBGLA53

[0127] The first step in the construction pathway of pGBGLA53 is the subcloning of two fragments, comprising the glaA promoter fused to almost entire coding sequence of the A.ficuum phytase gene. To achieve this, plasmid pGBGLA42 was digested with HindIII and EcoRI and the 1.8 kb HindIII/EcoRI 5'glaA promoter fragment was isolated by agarose gel-electrophoresis. Plasmid pFYT3 (European Patent Application 0 420 358 A1) was digested with EcoRI and BglII and the 1.6 kb EcoRI/BglII fragment comprising the 3'part of the glaA promoter fused to the 5' part of the phytase gene was isolated by agarose gel-electrophoresis and ligated together with the 1.8 kb HindIII/EcoRI 5'glaA promoter fragment isolated from pGBGLA42 into the HindIII and BglII sites of pSp73 (Promega). The resulting plasmid was designated pGBGLA49 (Figure 29).

[0128] The next step is the cloning of a 3'glaA non-coding region DNA fragment into pGBGLA49. Prior to cloning, this 3'glaA non-coding region DNA fragment (positioned at approximately 2.2 kb downstream the stop codon of the glaA gene) was amplified and provided with suitable restriction sites using the PCR method.

[0129] For this PCR reaction, the plasmid pAB6-1 was used as template and as primers two synthetic oligonucleotides with the following sequence were designed:

Oligo AB4234 (SEQ ID NO: 22):

5' GAAGACCCAGTCAAGCTTGCATGAGC 3'

(a 3'glaA non-coding sequence located approximately 2.2 kb downstream the stopcodon of the glaA gene)

Oligo AB 4235 (SEQ ID NO: 23):

5'TGACCAATTAAGCTTGCGGCCGCTCGAGGTCGCACCGGCAAAC 3'

(a 3'glaA non-coding sequence located approximately 4.4 kb downstream the stopcodon of the glaA gene)

[0130] Twenty-five amplification cycles (each: 1 minute 94°C; 1 minute 55°C; 1.5 minutes 72°C) were carried out in a DNA-amplifier (Perkin-Elmer). A schematic representation of this amplification is shown in figure 30A. The thus obtained fragment was digested with HindIII, purified by agarose gel-electrophoresis and subcloned into the HindIII site of pTZ19R. The resulting plasmid was designated pGBGLA47 (Figure 30).

[0131] Plasmid pGBGLA47 was digested with HindIII en NotI, the 2.2 kb 3"glaA non-coding DNA fragment was isolated by agarose gel-electrophoresis and cloned into the HindIII and NotI sites of pGBGLA49. The resulting plasmid was designated pGBGLA51 (Figure 31).

[0132] The last step in the construction pathway of pGBGLA53 is the cloning of the DNA fragment comprising the remaining part of the phytase coding sequence fused to the 3'glaA non-coding DNA fragment located just downstream the stop codon of the glaA gene. Prior to cloning, the remaining part of the phytase gene and the 3'glaA non-coding DNA fragment located just downstream the stopcodon of the glaA gene were fused and provided with suitable restriction sites using the PCR method. In the PCR, plasmid pAB6-1 was used as template and as primers two synthetic oligonucleotides were used, having the following sequences:

Oligo AB4236 (SEQ ID NO: 24):

5' TGACCAATAAAGCTTAGATCTGGGGGTGATTGGGCGGAGTGTTTTGCTT AGACAATCAATCCATTTCGC 3'

(36 bp of the phytase coding sequence, starting at the BglII site until the stopcodon fused to the 3'glaA non-coding region, starting just downstream the stopcodon of the glaA gene)

Oligo AB4233 (SEQ ID NO: 25):

5' TGACCAATAGATCTAAGCTTGACTGGGTCTTCTTGC 3'

(a 3'glaA non-coding sequence located approximately 2.2 kb downstream the stopcodon of the glaA gene)

[0133] Twenty-five amplification cycles (each: 1 minute 94°C; 1 minute 55°C; 1.5 minutes 72°C) were carried out in a DNA-amplifier (Perkin-Elmer). A schematic representation of this amplification is shown in figure 32A. The thus

obtained fragment was digested with HindIII, purified by agarose gel-electrophoresis and subcloned in both orientations into the HindIII site of pTZ19R. The resulting plasmids were designated pGBGLA48 and pGBGLA52 (figure 32B).

[0134] Plasmid pGBGLA52 was digested with BglII and partially digested with BamHI, the 2.2 kb phytase/3'glaA non-coding DNA fragment was isolated by agarose gel-electrophoresis and cloned into the BglII site of pGBGLA51. The derived plasmid with the 2.2 kb phytase/3'glaA non-coding DNA fragment in the correct orientation was designated pGBGLA53 (figure 33).

Transformation of A.niger GBA-107 with the vectors pGBGLA50 and pGBGLA53

[0135] Prior to transformation, E.coli sequences were removed from pGBGLA50 and pGBGLA53 by respectively XhoI or HindIII digestion followed by agarose gel-electrophoresis. The A.niger GBA-107 strain was transformed with respectively 1 µg pGBGLA50 fragment plus 1 µg pGBGLA53 fragment, 1 µg pGBGLA50 fragment plus 5 µg pGBGLA53 fragment, or 1 µg pGBGLA50 fragment plus 10 µg pGBGLA53 fragment using the transformation procedure described in the ' experimental section.

[0136] Single transformants were isolated, purified and Southern analysis was performed, using the same digests and probes as described in example 2, to verify integration of both pGBGLA50 and pGBGLA53. In about 10-20% of the analyzed transformants both pGBGLA50 and pGBGLA53 were integrated into the genome of the A.niger GBA-107 host strain. The transformant showing the correct integration pattern of a single copy pGBGLA50 and a single copy pGBGLA53, both integrated at the predefined 3'glaA non-coding region of the truncated glaA locus was used to remove subsequently the amdS selection marker gene.

Removal of the amdS marker gene by counter-selection on fluoracetamide containing plates

[0137] By performing the fluoracetamide counter-selection (as described in the experimental procedures), the amdS marker gene was deleted by an internal recombination event between the DNA repeats that were created by integration via a single cross-over event (i.e. the 3'glaA non-coding sequences). Proper removal of only the amdS marker gene was verified by Southern analysis using the same digests and probes as in example 2.

Example 4

Marker gene free introduction of the glaA gene and the phytase gene in A.oryzae

[0138] This example describes the marker gene free introduction of the glaA gene or the phytase gene in A.oryzae NRRL3485. A.oryzae NRRL3485 was transformed as described in the experimental section using the same vectors and approach as described in examples 2 and 3. Single transformants were isolated, purified and Southern analysis of chromosomal DNA of several transformants was performed to verify integrations of respectively the pGBGLA30 vector or the pGBGLA50 and pGBGLA53 vectors. In the Southern analysis, the same digests and probes were used as described in example 2.

Removal of the amdS gene by counter-selection on fluoracetamide containing plates

[0139] In case of integration of the pGBGLA30 vector, a transformant with a single copy of the pGBGLA30 integrated into the genome of the host strain A.oryzae NRRL3485 was used to remove the amdS gene properly. The counter-selection on fluoracetamide containing plates was performed as described in the experimental section. Correct removal of the amdS gene was verified by Southern analysis of chromosomal DNA of several fluoracetamide resistant strains. The same digests and probes were used as described in Example 2.

[0140] In case of co-transformation of the pGBGLA50 and pGBGLA53 vector, a transformant with a single copy of both pGBGLA50 and pGBGLA53 integrated into the host genome was used to remove the amdS marker gene properly. The counter-selection using fluoracetamide plates was performed as described in the experimental section. Correct removal of the amdS marker gene (e.g. the pGBGLA50 vector) was verified by Southern analysis of chromosomal DNA of several fluoracetamide resistant strains using the same digests and probes as described in example 2.

Example 5

Marker gene free introduction of the glaA gene and the phytase gene in T.reesei

[0141] This example describes the marker gene free introduction of the glaA gene or the phytase gene in Trichoderma reesei strain QM9414 (ATCC 26921). T.reesei QM9414 was transformed as described in the experimental section

using the same vectors and approach as described in examples 2 and 3. Single transformants were isolated, purified and Southern analysis of chromosomal DNA of several transformants was performed to verify whether integration of respectively the pGBGLA30 vector or the pGBGLA50 and pGBGLA53 vectors. In the Southern analysis, the same digests and probes were used as described in example 2.

Removal of the amdS gene by counter-selection on fluoracetamide containing plates

[0142] In case of integration of the pGBGLA30 vector, a transformant with a single copy of the pGBGLA30 integrated into the genome of the host strain T.reesei QM9414 was used to remove the amdS gene properly. The counter-selection on fluoracetamide containing plates was performed as described in the experimental section. Correct removal of the amdS gene was verified by Southern analysis of chromosomal DNA of several fluoracetamide resistant strains.

[0143] In case of co-transformation of the pGBGLA50 and pGBGLA53 vector, a transformant with a single copy of both pGBGLA50 and pGBGLA53 integrated into the host genome was used to remove the amdS marker gene properly. The counter-selection using fluoracetamide plates was performed as described in the experimental section. Correct removal of the amdS marker gene (e.g. the pGBGLA50 vector) was verified by Southern analysis on chromosomal DNA of several fluoracetamide resistant strains using the same digests and probes as described in example 2.

Example 6

Marker gene free introduction into P.chrysogenum of a P.chrysogenum gene by co-transformation using the amdS-gene as a selection marker

[0144] In this example the marker gene free introduction of a gene into the genome of P.chrysogenum by co-transformation is described.

[0145] In the co-transformation procedure, 2 different pieces of DNA are offered to the protoplasts, one of them being the amdS-selection marker, on the presence of which the first transformant selection takes place, as described in the experimental section, the second being another piece of DNA of interest, e.g. encoding a particular enzyme of interest. In a certain number of transformants both pieces of DNA will integrate into the chromosomes and will be stably maintained and expressed.

[0146] The amdS-selection marker gene can then be removed selectively from purified transformants by applying the counter-selection procedure as described in the experimental section, while the second piece of DNA will remain stably integrated into the chromosomes of the transformant. As an example to illustrate the general applicability of the method the stable, marker gene free introduction of a niaD-gene is described which enables a niaD-host to grow on nitrate as sole nitrogen-source.

[0147] Host for this co-transformation is a P.chrysogenum niaD-strain which lacks nitrate reductase and therefore is unable to grow on plates containing nitrate as sole nitrogen source. These strains can be easily obtained by well known procedures (Gouka et al., Journal of Biotechnology 20(1991), 189-200 and references there in)

[0148] During the co-transformation (procedure described in experimental section), two pieces of DNA are simultaneously offered to the protoplasts: the 7.6 kb EcoRI restriction fragment from pGBGLA28 containing the amdS selection marker gene and the 6.5 kb EcoRI restriction fragment from pPC1-1, containing the P.chrysogenum niaD-gene. Prior to transformation, both fragments have been separated from E.coli vector sequences by agarose gel-electrophoresis and purified from agarose gel by electro-elution. The first selection of transformants took place on selective plates containing acetamide as sole nitrogen source as described in the experimental section.

[0149] Among the transformants, co-transformants are found by replica plating spores of purified transformants to plates containing nitrate as sole nitrogen source. Typically about 20-60% of the replica plated transformants were able to grow on this medium, indicating that in these transformants not only the amdS selection marker gene but also the niaD-gene has integrated into the genome and is expressed.

Removal of the amdS gene by counter-selection on fluoracetamide containing plates

[0150] The amdS selection marker gene is subsequently removed from the co-transformants by counter-selection on fluoracetamide.

[0151] For direct selection on the amdS'/niaD$^+$-phenotype the medium used contained 10 mM fluor-acetamide. Spores were plated at a density of 10^4 spores per plate. After 5-7 days of incubation at 25°C, fluor-acetamide resistant colonies could be identified as solid colonies clearly distinct from the faint background. The niaD$^+$-phenotype of the recombinants is demonstrated by their growth on the fluoracetamide-medium containing nitrate as sole nitrogen source. The amdS'-phenotype of the recombinants was confirmed by lack of growth of the recombinants on plates containing acetamide as sole nitrogen source. Typically, 0.1-2% of the original number of plated spores exhibited the desired

phenotype.

[0152] Southern analysis on chromosomal DNA form several fluoracetamide resistant strains confirmed that the amdS selection marker gene was removed from the P.chrysogenum genome.

SEQUENCE LISTING

[0153]

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Gist-brocades B.V.
(B) STREET: Wateringseweg 1
(C) CITY: Delft
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 2611 XT

(ii) TITLE OF INVENTION: Selection Marker Gene Free Recombinant Strains: a Method for Obtaining Them and the Use of These Strains

(iii) NUMBER OF SEQUENCES: 37

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3100

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
CTAATCTAGA ATGCCTCAAT CCTGAA                                    26
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GACAGTCGAC AGCTATGGAG TCACCACA 28

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: TN0001

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCGATTAACT AGTTAA 16

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB2154

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AACCATAGGG TCGACTAGAC AATCAATCCA TTTCG 35

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB2155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTATTCGAA AGCTTATTCA TCCGGAGATC CTGAT 35

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB2994

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTGATAGAAT TCAGATCTGC AGCGGAGGCC TCTGTG 36

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3657

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGCTTGACGT CTACGTATTA ATGCGGCCGC T 31

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3658

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCGAAGCGGC CGCATTAATA CGTAGACGTC A 31

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3779

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AATTGGGGCC CATTAACTCG AGC 23

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3780

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AATTGCTCGA GTTAATGGGC CCC 23

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3448

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GTGCGAGGTA CCACAATCAA TCCATTTCGC 30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3449

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

ATGGTTCAAG AACTCGGTAG CCTTTTCCTT GATTCT 36

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3450

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AGAATCAAGG AAAAGGCTAC CGAGTTCTTG AACCAT 36

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3520

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ATCAATCAGA AGCTTTCTCT CGAGACGGGC ATCGGAGTCC CG 42

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3781

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

AATTGGGGCC CAGCGTCC 18

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3782

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AATTGGACGC TGGGCCCC 18

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3746

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TGACCAATAA AGCTTCTCGA GTAGCAAGAA GACCCAGTCA ATC 43

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3747

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CTACAAACGG CCACGCTGGA GATCCGCCGG CGTTCGAAAT AACCAGT 47

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB4234

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GAAGACCCAG TCAAGCTTGC ATGAGC 26

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB4235

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TGACCAATTA AGCTTGCGGC CGCTCGAGGT CGCACCGGCA AAC 43

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB4236

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TGACCAATAA AGCTTAGATC TGGGGGTGAT TGGGCGGAGT GTTTTGCTTA GACAATCAAT 60

CCATTTCGC 69

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB4233

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

TGACCAATAG ATCTAAGCTT GACTGGGTCT TCTTGC 36

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CTGCGAATTC GTCGACATGC CTCAATCCTG GG 32

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3515

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GGCAGTCTAG AGTCGACCTA TGGAGTCACC ACATTTC 37

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3701

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CTGCGAATTC GTCGACACTA GTGGTACCAT TATAGCCATA GGACAGCAAG 50

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3700

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GCTCTAGAGC GCGCTTATCA GCTTCCAGTT CTTCCCAGGA TTGAGGCATT TTTAATGTTA 60

CTTCTCTTGC 70

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3702

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CTGCGAATTC GTCGACACTA GTGGTACCAT CCTTTTGTTG TTTCCGGGTG 50

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3703

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GCTCTAGAGC GCGCTTATCA GCGGCCAGTT CTTCCCAGGA TTGAGGCATT GTATATGAGA 60
TAGTTGATTG 70

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3704

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GCTCTAGAAG TCGACACTAG TCTGCTACGT ACTCGAGAAT TTATACTTAG ATAAG 55

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TGCTCTAGAT CTCAAGCCAC AATTC 25

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3965

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CTGCTACGTA ATGTTTTCAT TGCTGTTTTA C 31

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3966

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CCGCCCAGTC TCGAGTCAGA TGGCTTTGGC CAGCCCC 37

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3825

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CGCGCTTATC AGCGGCCAGT TCTTCCCAGG ATTGAGGCAT ATGT 44

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(vii) IMMEDIATE SOURCE:

(B) CLONE: AB3826

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CTAGACATAT GCCTCAATCC TGGGAAGAAC TGGCCGCTGA TAAG 44

Claims

1. A vector containing a dominant, bidirectional and counter-selectable marker gene which is flanked by direct DNA repeats, which allow for internal recombination in the genome of a filamentous fungus.

2. A vector according to claim 1, wherein the direct DNA repeats are chosen from the 5' or 3' of the DNA sequence to be deleted from the genome.

3. A vector according to claim 1 or 2, wherein the selection marker gene is an acetamidase gene.

4. A vector according to claim 3, wherein the acetamidase gene is of fungal origin.

5. A vector according to claim 4, wherein the acetamidase gene is from an *Aspergillus* species.

6. A vector according to any one of claim 1 to 5, wherein the vector also contains a desired DNA fragment.

7. A vector according to claim 6, wherein the desired DNA fragment contains a genetic elements selected from the group consisting of: a gene, a cDNA, a promoter, a terminator, a regulatory element, an intron, a recognition sequence for a DNA-binding protein, a translation-initiation site, a restriction site and combinations thereof.

8. A vector according to claim 7, wherein the desired DNA fragment contains a sequence encoding a chymosin, a phytase, a xylanase, a lipase, an amylase, a protease, or a β-galactosidase.

9. A recombinant filamentous fungus transformed with the vector according to any one of claim 1 to 8.

10. A recombinant filamentous fungus according to claim 9, comprising at least two different vectors according to any one of claim 1 to 8.

11. A recombinant filamentous fungus according to claim 9 or 10, wherein the vector is integrated into the genome of said filamentous fungus through site-specific homologous recombination.

12. A recombinant filamentous fungus according to any one of claim 9 to 11, wherein the filamentous fungus is an *Aspergillus, Trichoderma,* or *Penicillium* species.

13. A method for obtaining a selection marker gene free recombinant filamentous fungus comprising the following steps:

    (a) integration into the genome of the filamentous fungus a desired DNA fragment and a dominant and bidirectional selection marker gene,
    (b) selection of the transformants,
    (c) deletion of the selection marker gene by recombination between repeats flanking the selection marker gene, and
    d) counter-selection based on the absence of the selection marker gene.

14. A method according to claim 13, **characterized in that** 5' or 3' of the selection marker gene a sequence is cloned which forms a repeat with a sequence which is 3' or 5' of the sequence to be deleted from the genome.

15. A method according to claim 13 or 14, wherein the desired DNA fragment contains a genetic elements selected from the group consisting of: a gene, a cDNA, a promoter, a terminator, a regulatory element, an intron, a recognition sequence for a DNA-binding protein, a translation-initiation site, a restriction site and combinations thereof.

16. A method according to any one of claim 13 to 14, wherein steps a) to d) are repeated on the recombinant filamentous fungus obtained, using either the same or a different desired DNA fragment.

17. A method according to any one of claims 13 to 16, wherein the selection marker gene is an acetamidase gene.

18. A method according to claim 17, wherein the selection marker gene is an acetamidase gene is of fungal origin.

19. A method according to claim 18, wherein the selection marker gene is an acetamidase gene from an *Aspergillus* species.

20. A method for the production of a bio-active compound, which method comprises the step of culturing a filamentous fungus produced according to the method of any one of claims 13 to 19.

21. A method according to claim 20, wherein the bio-active compound is a protein.

22. A method according to claim 20, wherein the bio-active compound is an antibiotic.

23. Use of a filamentous fungus produced by the method of any one of claims 13 to 19 for the production of a bio-active compound.

24. Use according to claim 23, wherein the bio-active compound is a protein.

25. Use according to claim 23, wherein the bio-active compound is an antibiotic.

Patentansprüche

1. Vektor, enthaltend ein dominantes, bidirektionales und gegenselektierbares Markergen, das von direkten DNA-Wiederholungen (repeats) flankiert ist, die eine interne Rekombination im Genom eines filamentösen Pilzes ermöglichen.

2. Vektor nach Anspruch 1, wobei die direkten DNA-Wiederholungen unter den 5'- oder 3'-DNA-Sequenzen, die vom Genom zu deletieren sind, ausgewählt sind.

3. Vektor nach Anspruch 1 oder 2, wobei es sich beim Selektionsmarkergen um ein Acetamidase-Gen handelt.

4. Vektor nach Anspruch 3, wobei das Acetamidase-Gen pilzlichen Ursprungs ist.

5. Vektor nach Anspruch 4, wobei das Acetamidase-Gen von einer Aspergillus-Spezies stammt.

6. Vektor nach einem der Ansprüche 1 bis 5, wobei der Vektor ferner ein gewünschtes DNA-Fragment enthält.

7. Vektor nach Anspruch 6, wobei das gewünschte DNA-Fragment ein genetisches Element enthält, das aus folgender Gruppe ausgewählt ist: ein Gen, eine cDNA, ein Promotor, ein Terminator, ein regulatorisches Element, ein Intron, eine Erkennungssequenz für ein DNA-bindendes Protein, eine Translationsinitiationsstelle, eine Restriktionsstelle und Kombinationen davon.

8. Vektor nach Anspruch 7, wobei das gewünschte DNA-Fragment eine Sequenz enthält, die für ein Chymosin, eine Phytase, eine Xylanase, eine Lipase, eine Amylase, eine Protease oder eine β-Galactosidase kodiert.

9. Rekombinanter, filamentöser Pilz, transformiert mit dem Vektor gemäß einem der Ansprüche 1 bis 8.

10. Rekombinanter, filamentöser Pilz nach Anspruch 9, umfassend mindestens zwei verschiedene Vektoren gemäß einem der Ansprüche 1 bis 8.

11. Rekombinanter, filamentöser Pilz nach Anspruch 9 oder 10, wobei der Vektor durch ortsspezifische, homologe Rekombination in das Genom des filamentösen Pilzes integriert ist.

12. Rekombinanter, filamentöser Pilz nach einem der Ansprüche 9 bis 11, wobei es sich beim filamentösen Pilz um eine Aspergillus-, Trichoderma- oder Penicillium-Spezies handelt.

13. Verfahren zum Erhalt eines rekombinanten, filamentösen Pilzes, der frei von einem Selektionsmarkergen ist, umfassend die folgenden Stufen:

    (a) Integrieren eines gewünschten DNA-Fragments und eines dominanten und bidirektionalen Selektions-

markergens in das Genom des filamentösen Pilzes,

(b) Auswählen der Transformanten,

(c) Deletion des Selektionsmarkergens durch Rekombination zwischen Wiederholungen, die das Selektionsmarkergen flankieren, und

(d) Gegenselektion, auf der Grundlage der Abwesenheit des Selektionsmarkergens.

14. Verfahren nach Anspruch 13, **dadurch gekennzeichnet, dass** in 5'- oder 3'-Stellung des Selektionsmarkergens eine Sequenz geklont ist, die eine Wiederholung mit einer Sequenz bildet, die sich in 3'- oder 5'-Stellung von der aus dem Genom zu deletierenden Sequenz befindet.

15. Verfahren nach Anspruch 13 oder 14, wobei das gewünschte DNA-Fragment ein genetisches Element enthält, das aus folgender Gruppe ausgewählt ist: ein Gen, eine cDNA, ein Promotor, ein Terminator, ein regulatorisches Element, ein Intron, eine Erkennungssequenz für ein DNA-bindendes Protein, eine Translationsinitiationsstelle, eine Restriktionsstelle und Kombinationen davon.

16. Verfahren nach einem der Ansprüche 13 bis 14, wobei die Stufen a) bis d) am erhaltenen rekombinanten, filamentösen Pilz wiederholt werden, wobei man entweder das gleiche oder ein unterschiedliches gewünschtes DNA-Fragment verwendet.

17. Verfahren nach einem der Ansprüche 13 bis 16, wobei es sich beim Selektionsmarkergen um ein Acetamidase-Gen handelt.

18. Verfahren nach Anspruch 17, wobei es sich beim Selektionsmarkergen um ein Acetamidase-Gen pilzlichen Ursprungs handelt.

19. Verfahren nach Anspruch 18, wobei es sich beim Selektionsmarkergen um ein Acetamidase-Gen aus einer Aspergillus-Spezies handelt.

20. Verfahren zur Herstellung einer biologisch aktiven Verbindung, wobei das Verfahren die Stufe der Züchtung eines filamentösen Pilzes umfasst, der gemäß dem Verfahren nach einem der Ansprüche 13 bis 19 erzeugt worden ist.

21. Verfahren nach Anspruch 20, wobei es sich bei der biologisch aktiven Verbindung um ein Protein handelt.

22. Verfahren nach Anspruch 20, wobei es sich bei der biologisch aktiven Verbindung um ein Antibiotikum handelt.

23. Verwendung eines filamentösen Pilzes, erzeugt durch das Verfahren nach einem der Ansprüche 13 bis 19, zur Herstellung einer biologisch aktiven Verbindung.

24. Verwendung nach Anspruch 23, wobei es sich bei der biologisch aktiven Verbindung um ein Protein handelt.

25. Verwendung nach Anspruch 23, wobei es sich bei der biologisch aktiven Verbindung um ein Antibiotikum handelt.

Revendications

1. Un vecteur contenant un gène de marqueur de sélection dominant bidirectionnel et qui peut être contre-sélectionné qui est encadré par des répétitions directes d'ADN qui permettent la recombinaison interne du gène de marqueur au sein du génome du champignon filamenteux.

2. Un vecteur selon la revendication 1, **caractérisé en ce que** les répétitions directes d'ADN sont choisies parmi une séquence qui est en 5' ou en 3' de la séquence d'ADN à supprimer du génome.

3. Un vecteur selon l'une quelconque des revendications 1 à 2, **caractérisé en ce que** le gène de marqueur de sélection est un gène d'acétamidase.

4. Un vecteur selon la revendication 3, **caractérisé en ce que** le gène d'acétamidase provient d'un champignon.

5. Un vecteur selon la revendication 4, **caractérisé en ce que** le gène d'acétamidase provient d'une espèce d'*Aspergillus.*

6. Un vecteur selon l'une quelconque des revendications 1 à 5, **caractérisé en ce que** le vecteur contient également un fragment d'ADN désiré.

7. Un vecteur selon la revendication 6, **caractérisé en ce que** le fragment d'ADN désiré contient un élément génétique sélectionné parmi le groupe constitué par : un gène, un ADN complémentaire, un promoteur, un terminateur, un élément régulateur, un intron, une séquence de reconnaissance pour une protéine de liaison à l'ADN, un site d'initiation de traduction, un site de restriction et combinaisons de ces éléments.

8. Un vecteur selon la revendication 7, **caractérisé en ce que** le fragment d'ADN désiré contient une séquence encodant une chymosine, une phytase, une xylanase, une lipase, une amylase, une protéase, ou une β-galactosidase.

9. Un champignon filamenteux recombinant transformé avec le vecteur selon l'une quelconque des revendications 1 à 8.

10. Un champignon filamenteux recombinant selon la revendication 9, **caractérisé en ce qu'**il contient au moins deux vecteurs selon l'une quelconque des revendications 1 à 8.

11. Un champignon filamenteux recombinant selon l'une quelconque des revendications 9 à 10, **caractérisé en ce que** le vecteur est intégré au sein du génome du dit champignon filamenteux via recombinaison homologue à un site spécifique.

12. Un champignon filamenteux recombinant selon l'une quelconque des revendications 9 à 11, **caractérisé en ce que** le champignon filamenteux est une espèce d'*Aspergillus,* de *Trichoderma* ou de *Pénicillium.*

13. Une méthode pour obtenir un champignon filamenteux recombinant qui ne possède pas de marqueur de sélection; cette dite méthode comprenant les étapes suivantes :

    (a) intégration d'un fragment d'ADN désiré et d'un gène de marqueur de sélection dominant et bidirectionnel dans le génome du champignon filamenteux,
    (b) sélection des transformants,
    (c) délétion du gène de marqueur de sélection par recombinaison entre les répétitions encadrant le gène de marqueur de sélection,
    (d) contre-sélection basée sur l'absence du gène de marqueur de sélection.

14. Une méthode selon la revendication 13, **caractérisée en ce qu'**en 5'ou 3' du gène de marqueur de sélection une séquence est clonée qui forme une répétition avec une séquence qui est en 3'ou 5' de la séquence à être délétée du génome.

15. Une méthode selon l'une quelconque des revendications 13 à 14, **caractérisée en ce que** le fragment d'ADN désiré contient un élément génétique sélectionné parmi le groupe constitué par : un gène, un ADN complémentaire, un promoteur, un terminateur, un élément régulateur, un intron, une séquence de reconnaissance pour une protéine de liaison à l'ADN, un site d'initiation de traduction, un site de restriction et combinaisons de ces éléments.

16. Une méthode selon l'une quelconque des revendications 13 à 14, **caractérisée en ce que** les étapes (a) à (d) sont répétées sur le champignon filamenteux recombinant obtenu, en utilisant le même ou bien un fragment d'ADN désiré différent.

17. Une méthode selon l'une quelconque des revendications 13 à 16, **caractérisée en ce que** le gène de marqueur de sélection est un gène d'acétamidase.

18. Une méthode selon la revendication 17, **caractérisé en ce que** le gène de marqueur de sélection est un gène d'acétamidase qui provient d'un champignon.

19. Une méthode selon la revendication 18, **caractérisée en ce que** le gène de marqueur de sélection est un gène d'acétamidase qui provient d'une espèce d'*Aspergillus.*

20. Une méthode pour la production d'un composé bio-actif, **caractérisée en ce que** la méthode comprend l'étape de cultiver le champignon filamenteux produit selon la méthode selon l'une quelconque des revendications 13 à 19.

21. Une méthode selon la revendication 20, **caractérisée en ce que** le composé bio-actif est une protéine.

22. Une méthode selon la revendication 20, **caractérisée en ce que** le composé bio-actif est un antibiotique.

23. Utilisation du champignon filamenteux produit selon la méthode selon l'une quelconque des revendications 13 à 19 pour la production d'un composé bio-actif.

24. Utilisation selon la revendication 23, **caractérisée en ce que** le composé bio-actif est une protéine.

25. Utilisation selon la revendication 23, **caractérisée en ce que** le composé bio-actif est un antibiotique.

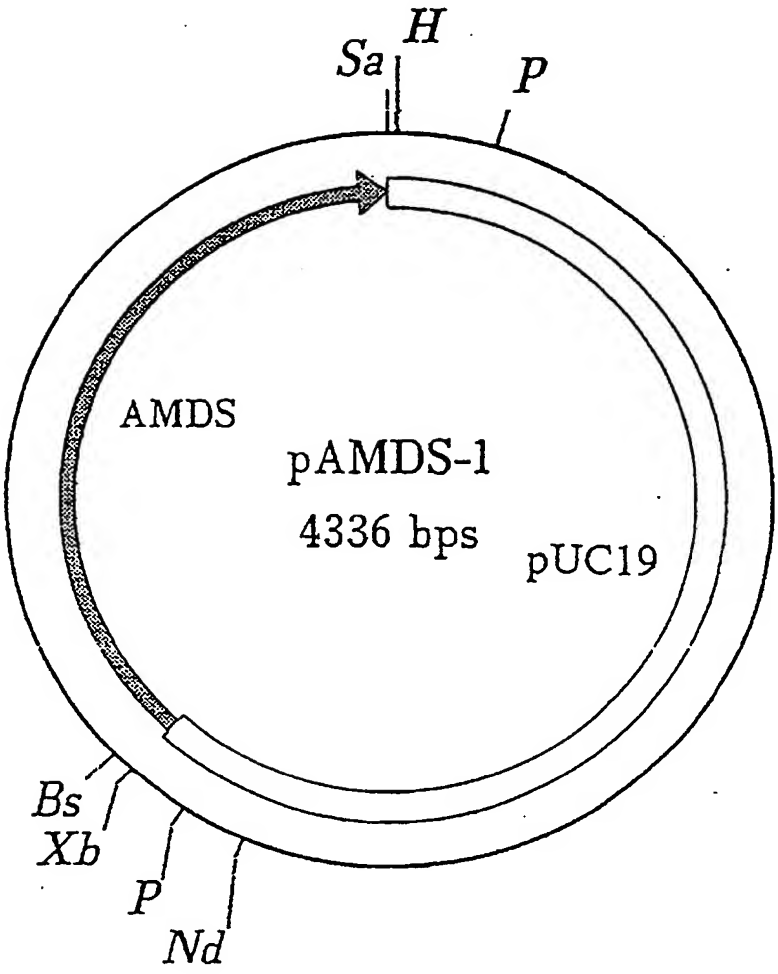


Fig. 1

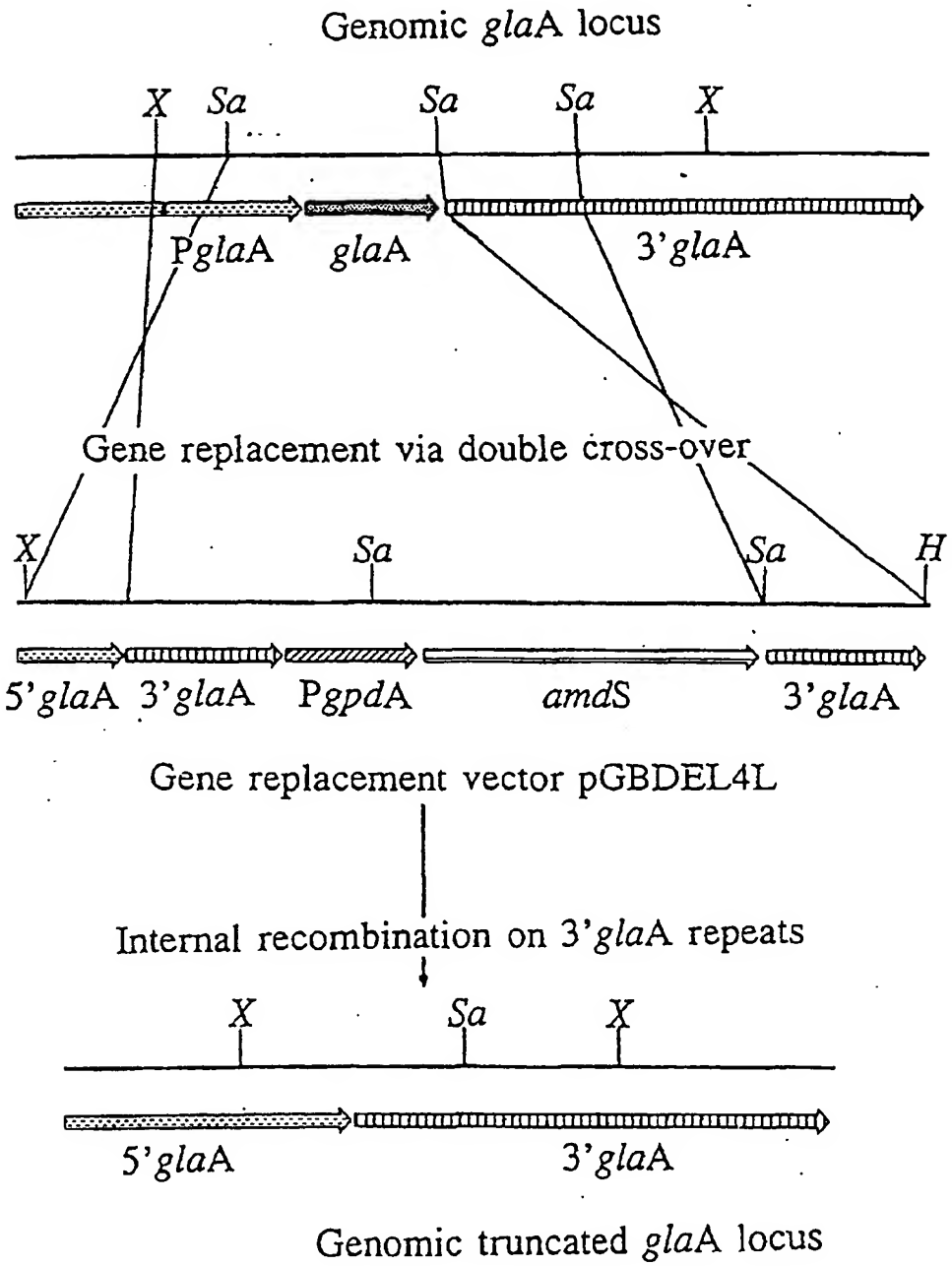

Genomic glaA locus
X
Sa
Sa
Sa
X
PglaA
glaA
3'glaA
Gene replacement via double cross-over
X
Sa
Sa
H
5'glaA
3'glaA
PgpdA
amdS
3'glaA
Gene replacement vector pGBDEL4L
Internal recombination on 3'glaA repeats
X
Sa
X
5'glaA
3'glaA
Genomic truncated glaA locus

Fig. 2

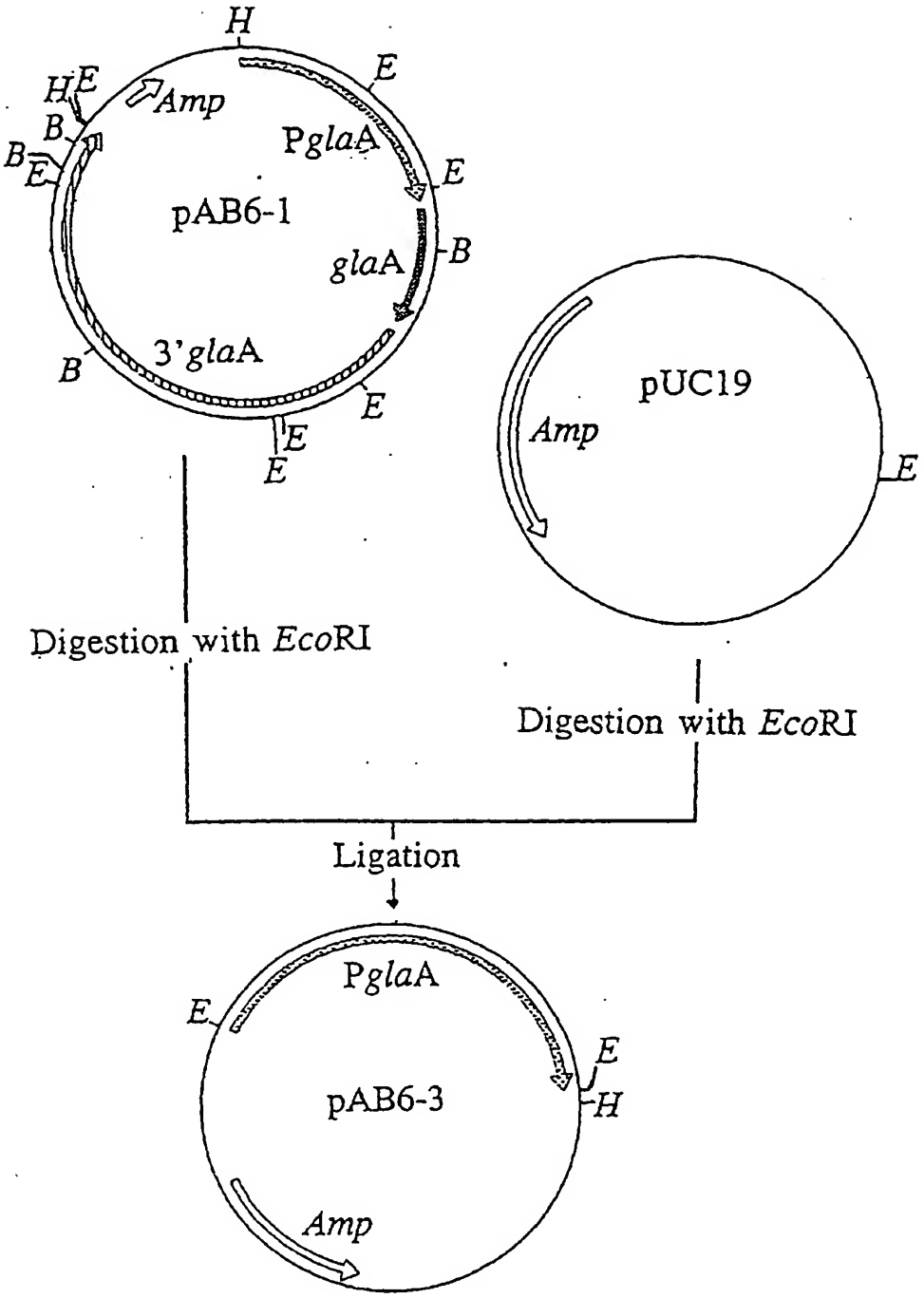

H
E
Amp
PglaA
E
pAB6-1
glaA
B
3'glaA
B
E
E
E
H
B
B
E
pUC19
Amp
E
Digestion with EcoRI
Digestion with EcoRI
Ligation
PglaA
E
E
pAB6-3
H
Amp

Fig. 3a

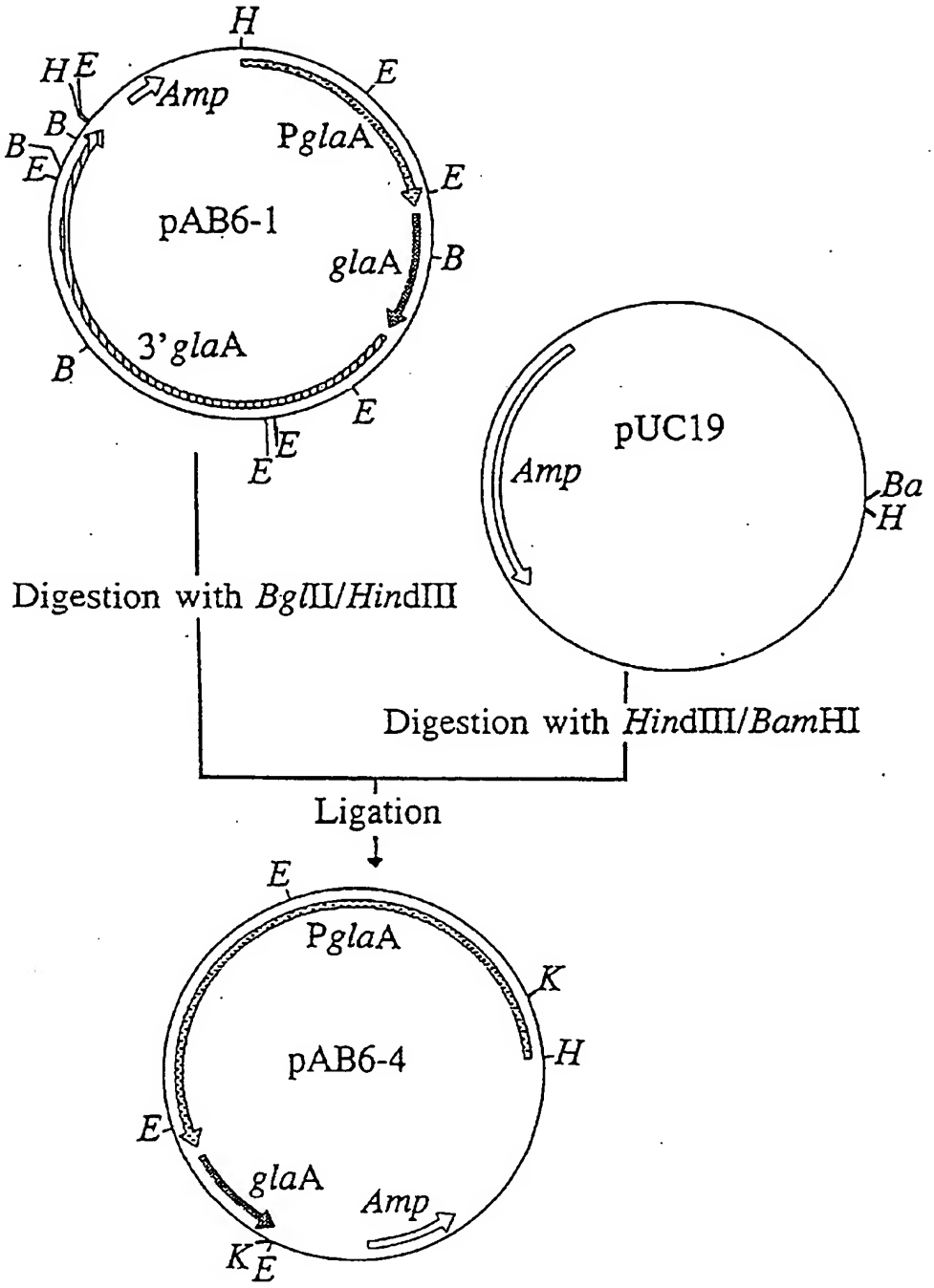

H
H E
Amp
E
B
PglaA
B
E
E
pAB6-1
glaA
B
3'glaA
B
E
E
E
pUC19
Amp
Ba
H
Digestion with BglII/HindIII
Digestion with HindIII/BamHI
Ligation
E
PglaA
K
pAB6-4
H
E
glaA
Amp
K
E

Fig. 3b

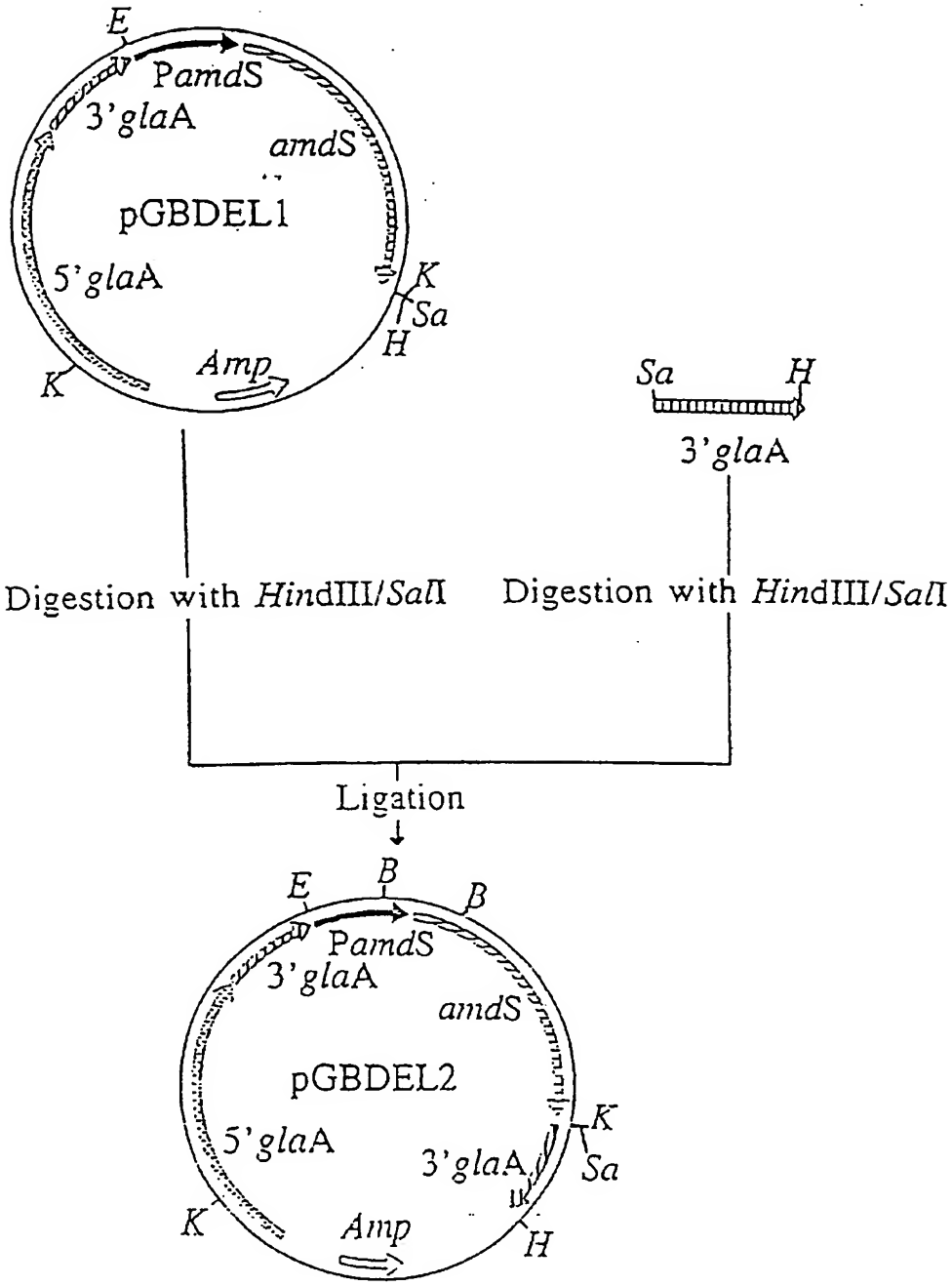

E
PamdS
3'glaA
amdS
pGBDEL1
5'glaA
K
Sa
H
Amp
K
Sa
H
3'glaA
Digestion with HindIII/SalI
Digestion with HindIII/SalI
Ligation
E
B
B
PamdS
3'glaA
amdS
pGBDEL2
5'glaA
3'glaA
K
Sa
K
Amp
H

Fig. 4

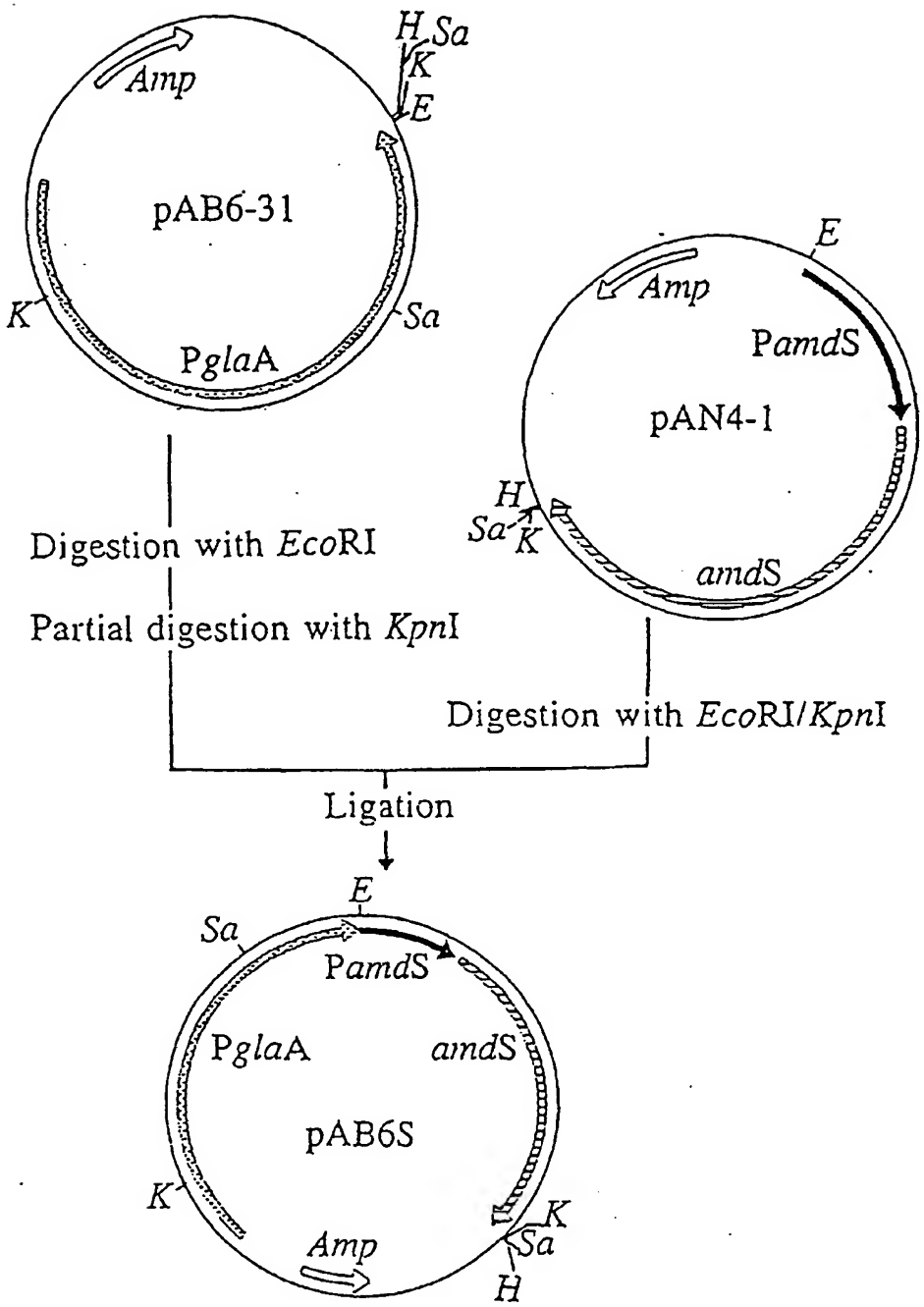

H Sa
K
E
Amp
pAB6-31
K
Sa
PglaA
E
Amp
PamdS
pAN4-1
H
Sa
K
amdS
Digestion with EcoRI
Partial digestion with KpnI
Digestion with EcoRI/KpnI
Ligation
E
Sa
PamdS
PglaA
amdS
pAB6S
K
Amp
K
Sa
H

Fig. 5

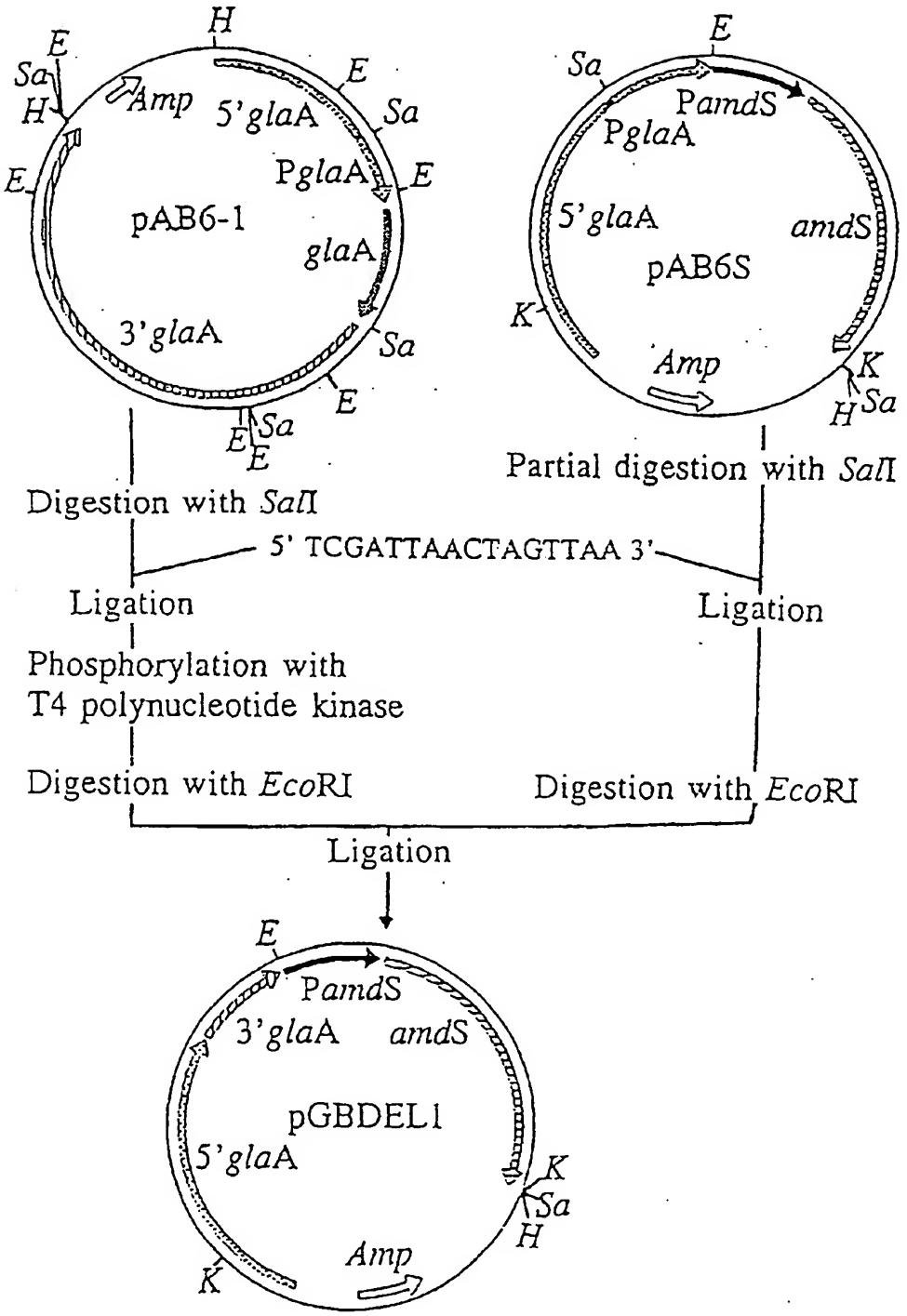

pAB6-1
5'glaA
PglaA
glaA
3'glaA
Amp
pAB6S
PamdS
amdS
Digestion with SalI
Partial digestion with SalI
5' TCGATTAACTAGTTAA 3'
Ligation
Ligation
Phosphorylation with
T4 polynucleotide kinase
Digestion with EcoRI
Digestion with EcoRI
Ligation
pGBDEL1

Fig. 6

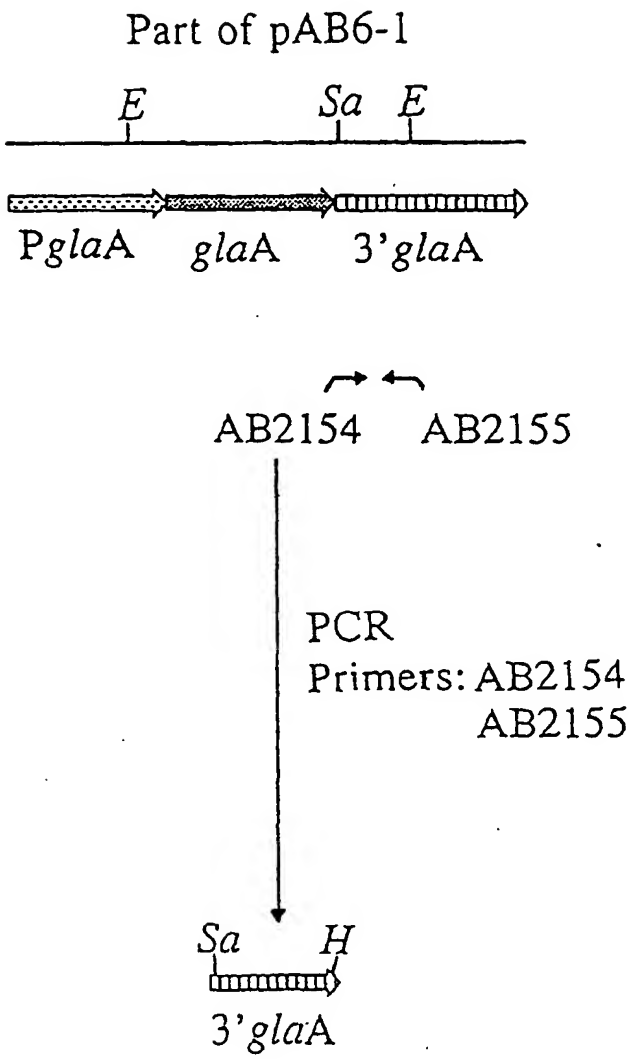

Part of pAB6-1
E
Sa
E
PglaA
glaA
3'glaA
AB2154
AB2155
PCR
Primers: AB2154
AB2155
Sa
H
3'glaA

Fig. 7a

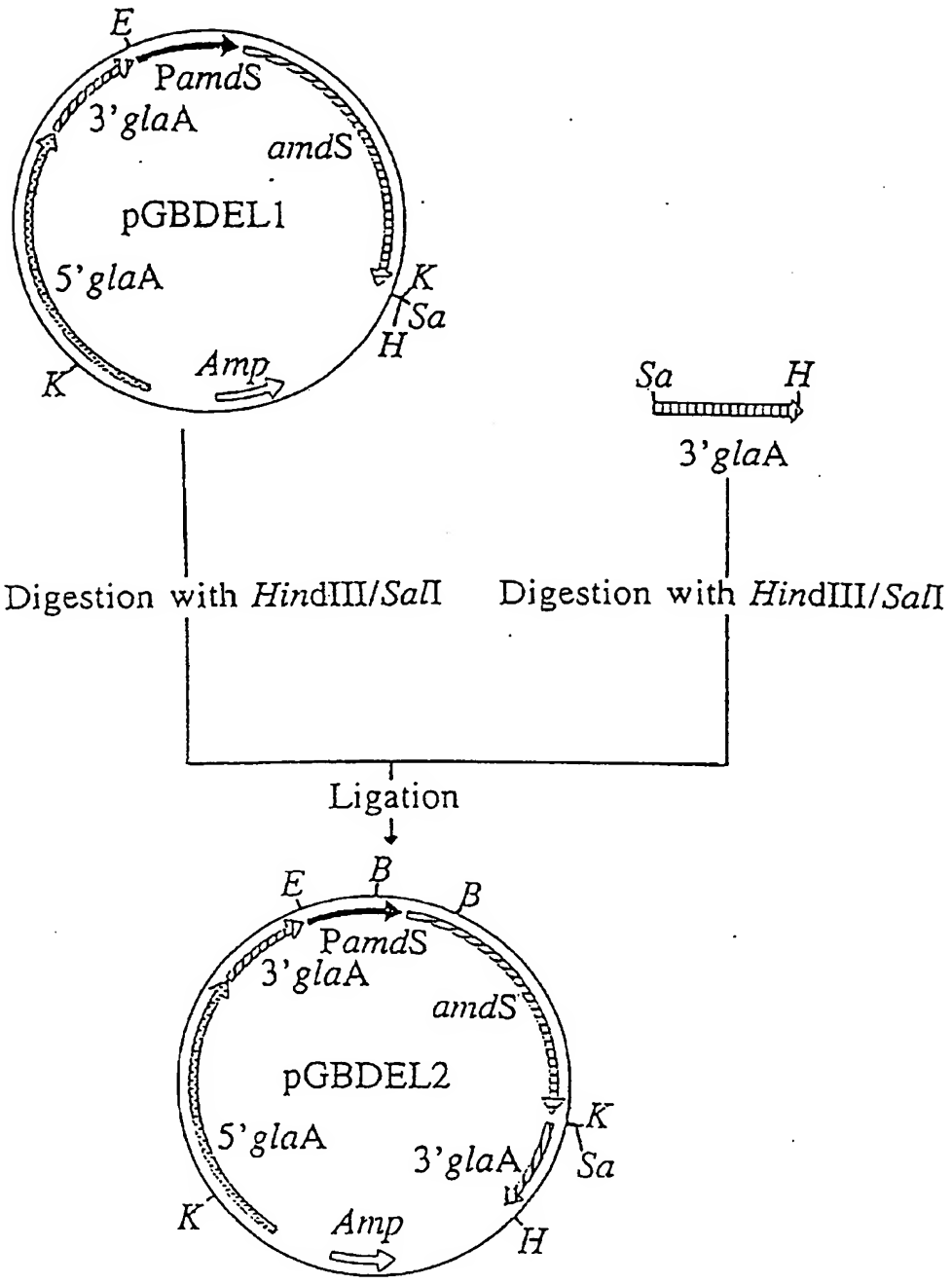

E
PamdS
3'glaA
amdS
pGBDEL1
5'glaA
K
Sa
H
Amp
K
Sa
H
3'glaA
Digestion with HindIII/SalI
Digestion with HindIII/SalI
Ligation
E
B
B
PamdS
3'glaA
amdS
pGBDEL2
5'glaA
3'glaA
K
Sa
K
Amp
H

Fig. 7b

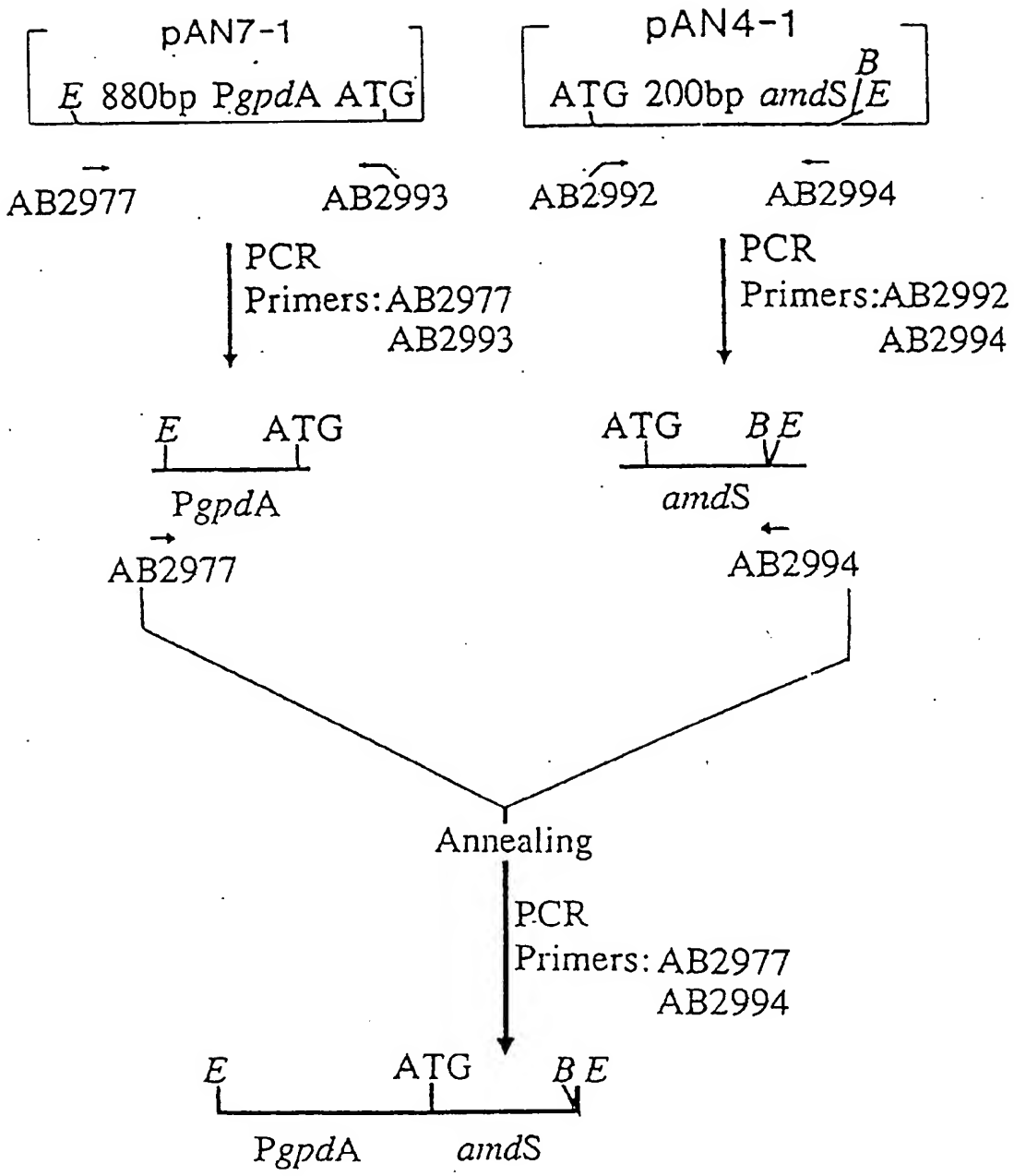

pAN7-1
E 880bp PgpdA ATG
pAN4-1
ATG 200bp amdS B E
AB2977
AB2993
AB2992
AB2994
PCR
Primers: AB2977
AB2993
PCR
Primers: AB2992
AB2994
E ATG
PgpdA
ATG B E
amdS
AB2977
AB2994
Annealing
PCR
Primers: AB2977
AB2994
E ATG B E
PgpdA amdS

Fig. 8a

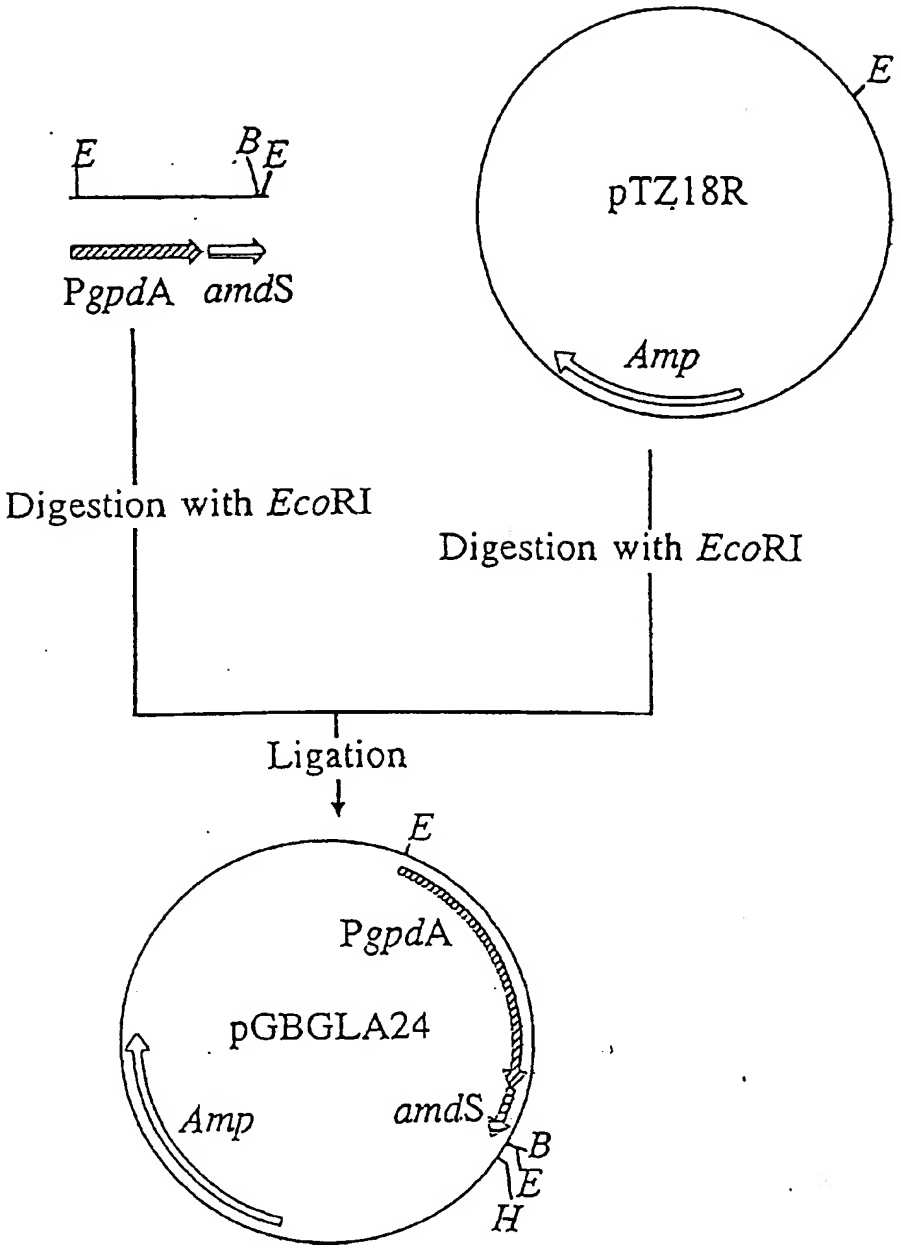

E
B
E
PgpdA amdS
pTZ18R
E
Amp
Digestion with EcoRI
Digestion with EcoRI
Ligation
E
PgpdA
pGBGLA24
Amp
amdS
B
E
H

Fig. 8b

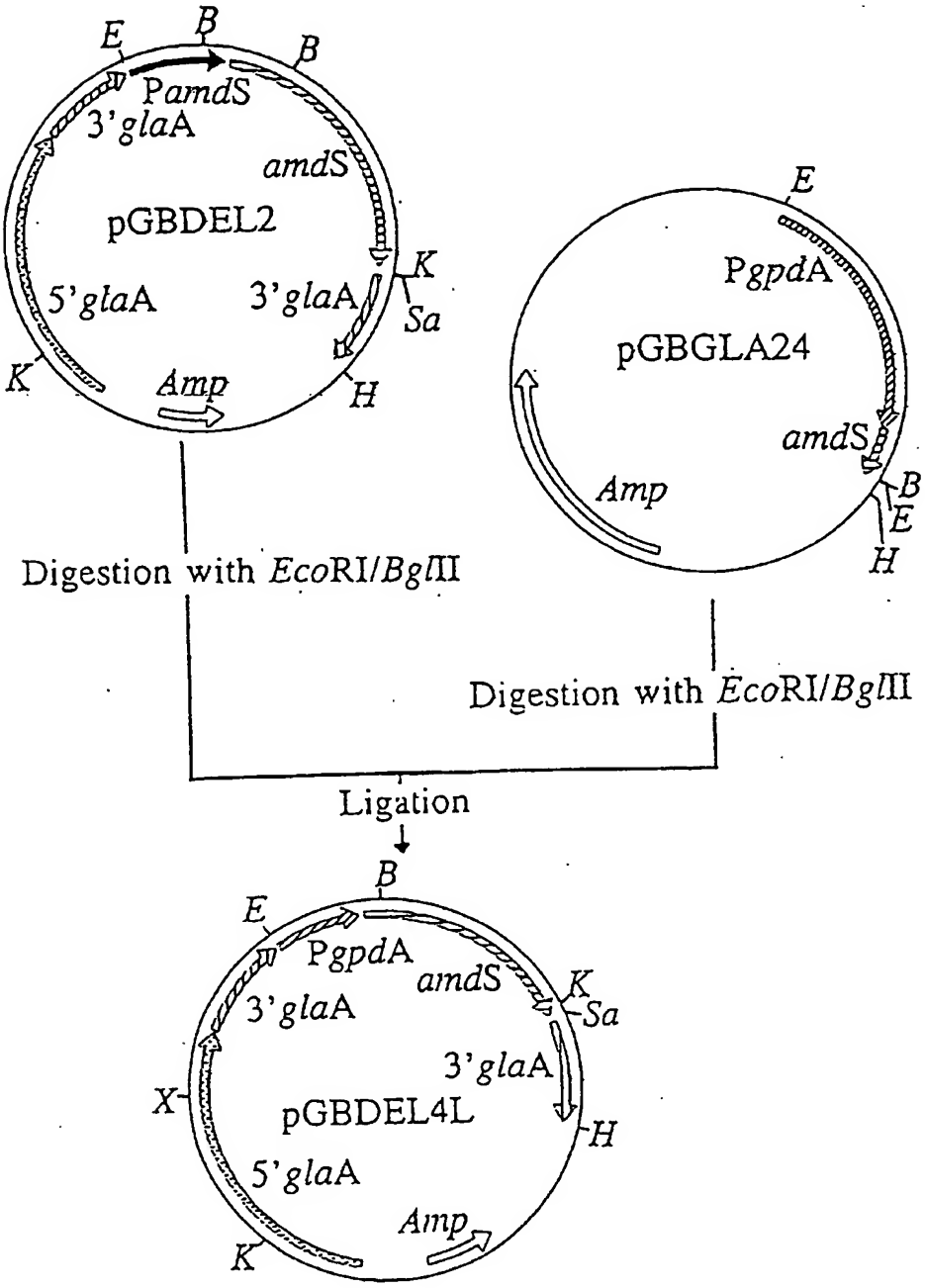
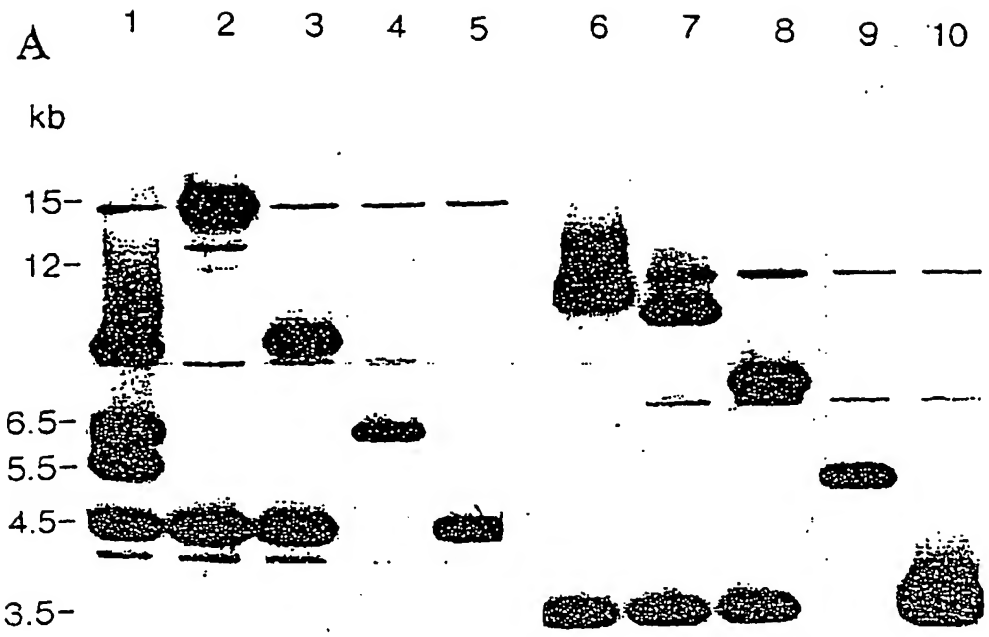

E
B
B
PamdS
3'glaA
amdS
pGBDEL2
K
Sa
5'glaA
3'glaA
K
Amp
H
E
PgpdA
pGBGLA24
amdS
Amp
B
E
H
Digestion with EcoRI/BglII
Digestion with EcoRI/BglII
Ligation
B
E
PgpdA
amdS
K
Sa
3'glaA
3'glaA
X
pGBDEL4L
H
5'glaA
Amp
K

Fig. 9



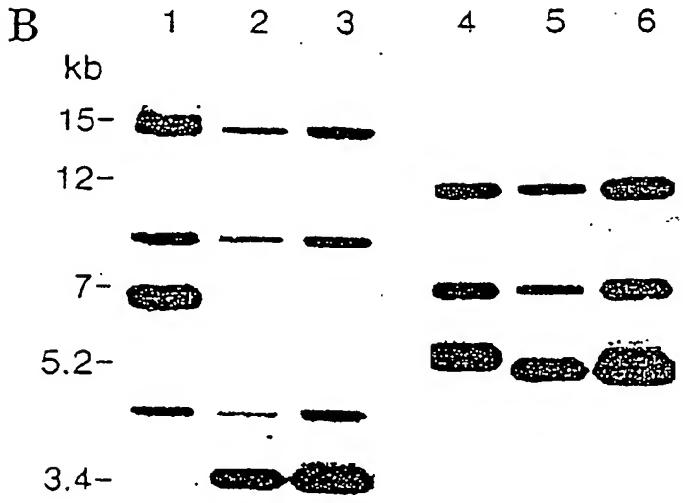


Fig. 10a

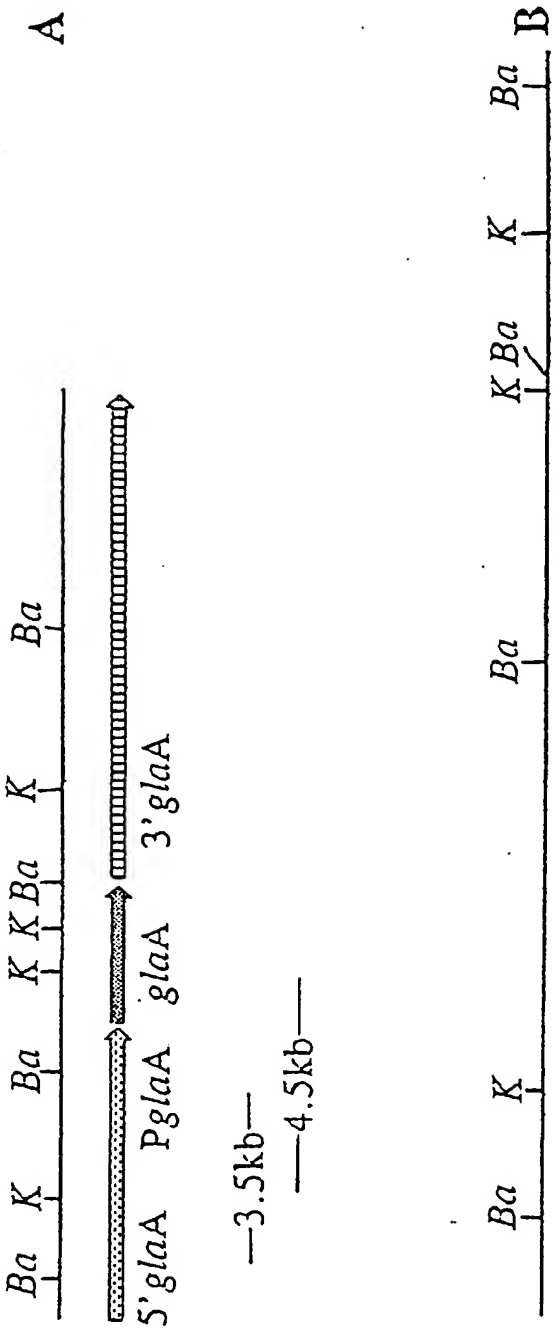

A
Ba K Ba K K Ba K Ba
5'glaA PglaA glaA 3'glaA
3.5kb
4.5kb
B
Ba K Ba K Ba K Ba
5'glaA 3'glaA PgpdA amdS 3'glaA
5.5kb
6.3kb

Fig. 10b

C

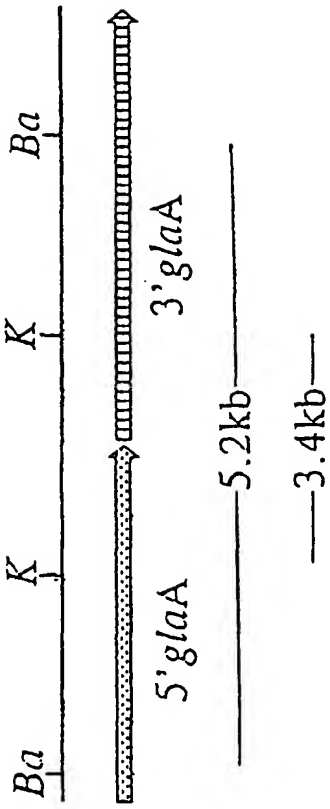


Fig. 11

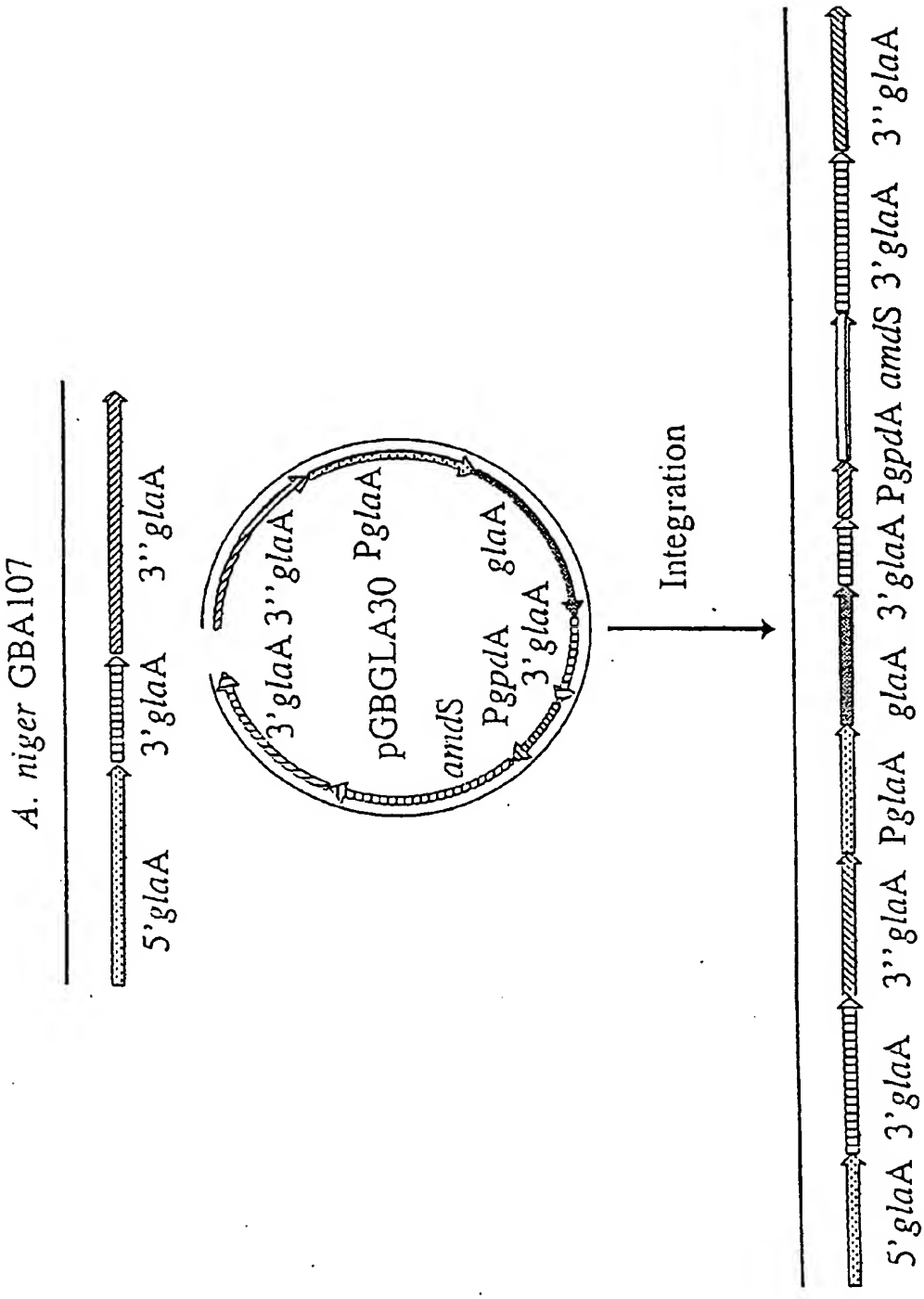

A. niger GBA107
5'glaA
3'glaA
3''glaA
pGBGLA30
3'glaA 3''glaA
PglaA
amdS
PgpdA
glaA
3'glaA
Integration
5'glaA 3'glaA
3''glaA
PglaA
glaA
3'glaA
PgpdA
amdS
3'glaA
3''glaA

Fig. 12a

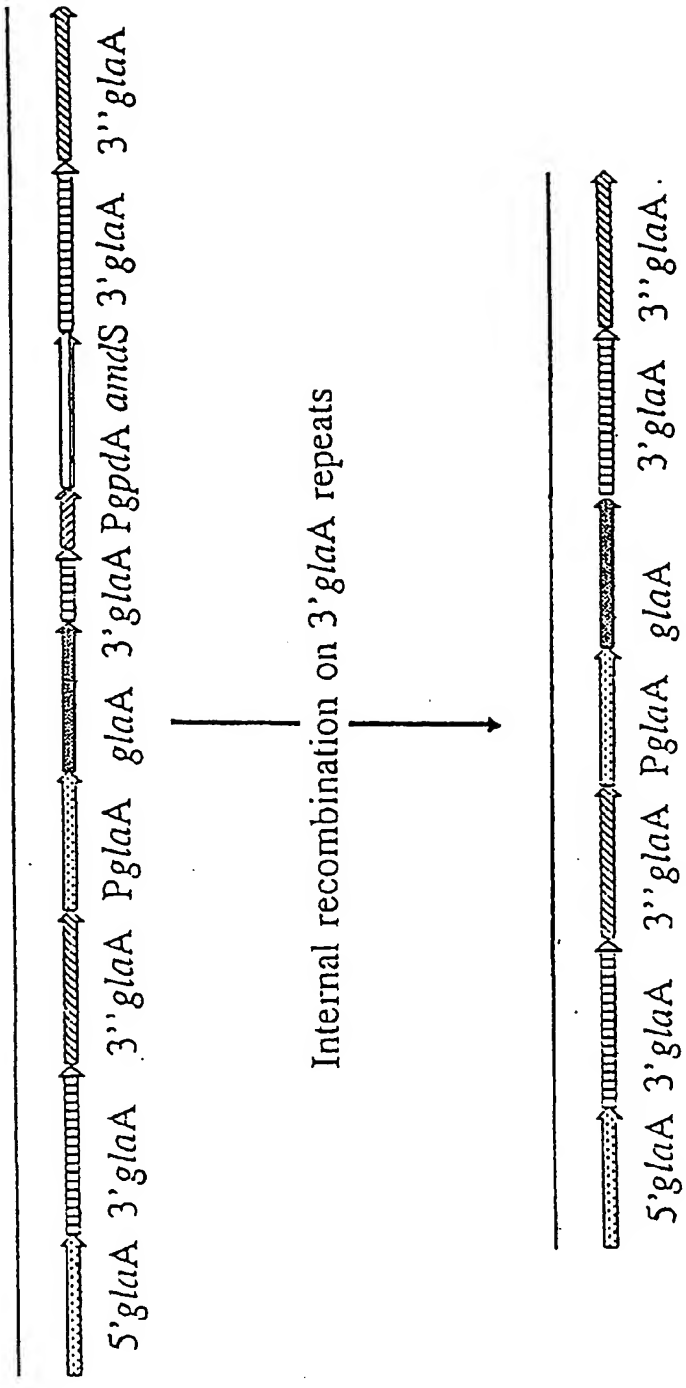

5'glaA 3'glaA 3''glaA PglaA glaA 3'glaA PgpdA amdS 3'glaA 3''glaA
Internal recombination on 3'glaA repeats
5'glaA 3'glaA 3''glaA PglaA glaA 3'glaA 3''glaA

Fig. 12b

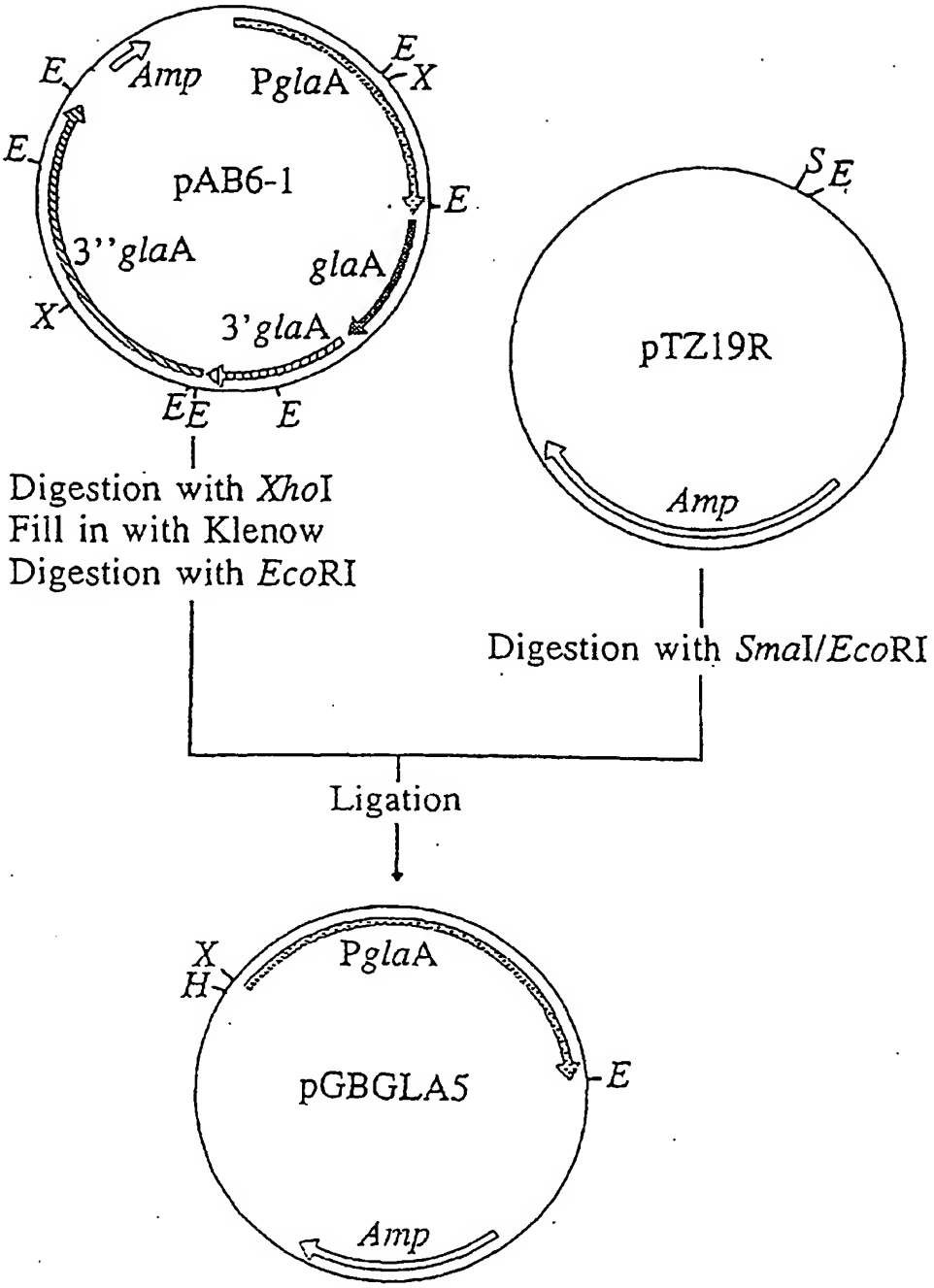

E
Amp
PglaA
E
X
E
pAB6-1
E
3''glaA
glaA
X
3'glaA
E
E
E
S
E
pTZ19R
Amp
Digestion with XhoI
Fill in with Klenow
Digestion with EcoRI
Digestion with SmaI/EcoRI
Ligation
X
H
PglaA
pGBGLA5
E
Amp

Fig. 13

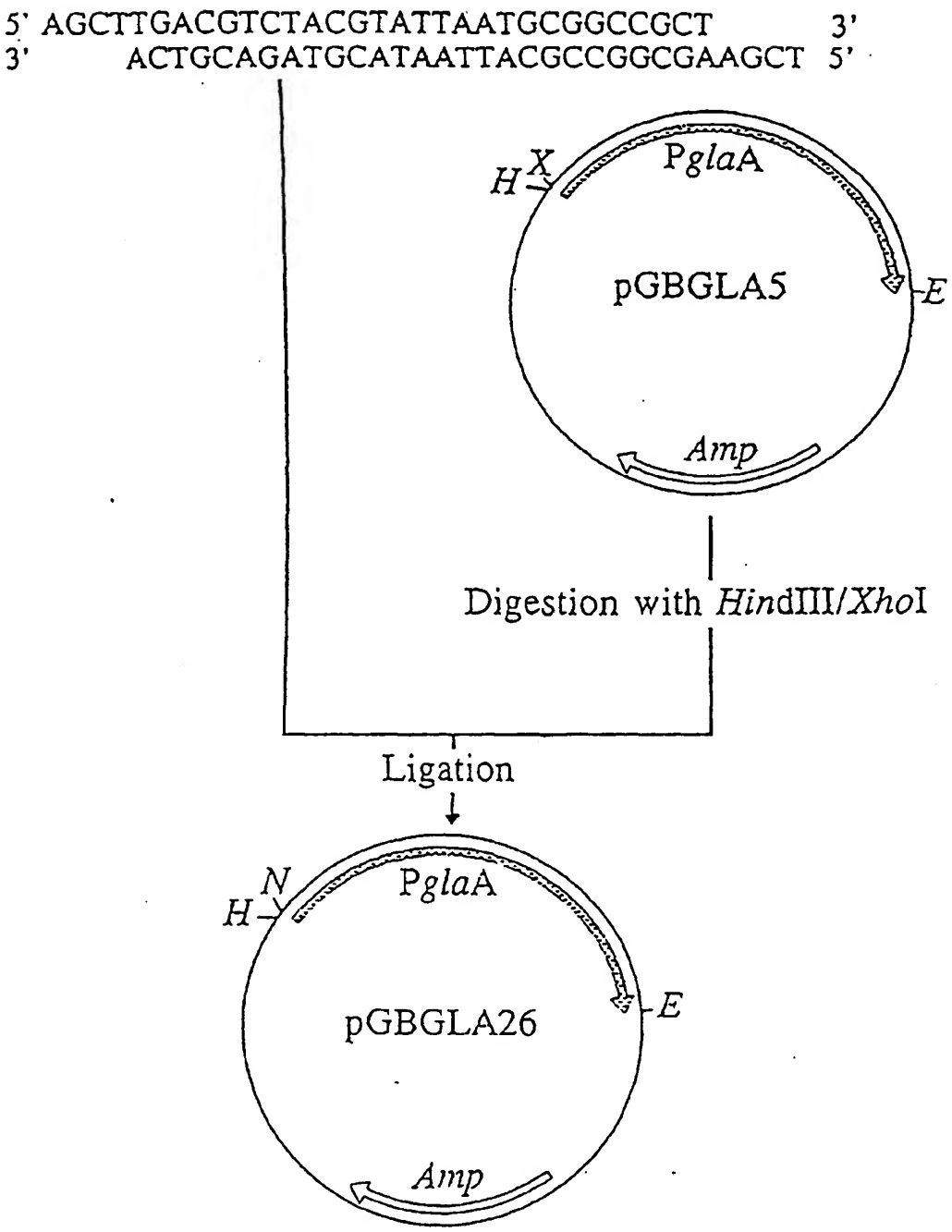

5' AGCTTGACGTCTACGTATTAATGCGGCCGCT 3'
3' ACTGCAGATGCATAATTACGCCGGCGAAGCT 5'
H
X
PglaA
pGBGLA5
E
Amp
Digestion with HindIII/XhoI
Ligation
N
H
PglaA
pGBGLA26
E
Amp

Fig. 14

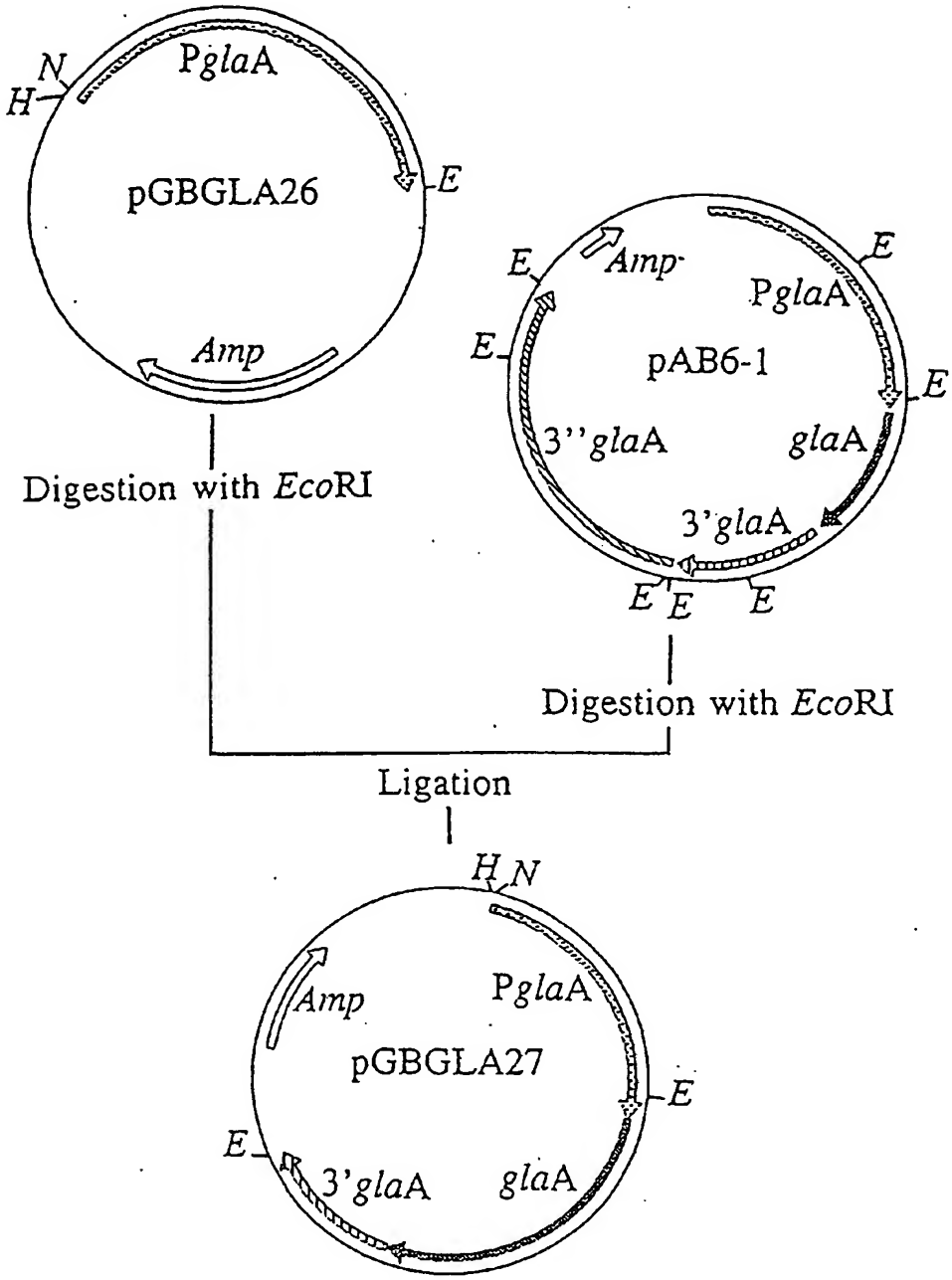

N
H
PglaA
pGBGLA26
E
Amp
Digestion with EcoRI
E
Amp
E
PglaA
E
pAB6-1
E
3''glaA
glaA
3'glaA
E
E
E
Digestion with EcoRI
Ligation
H
N
Amp
PglaA
pGBGLA27
E
E
3'glaA
glaA

Fig. 15

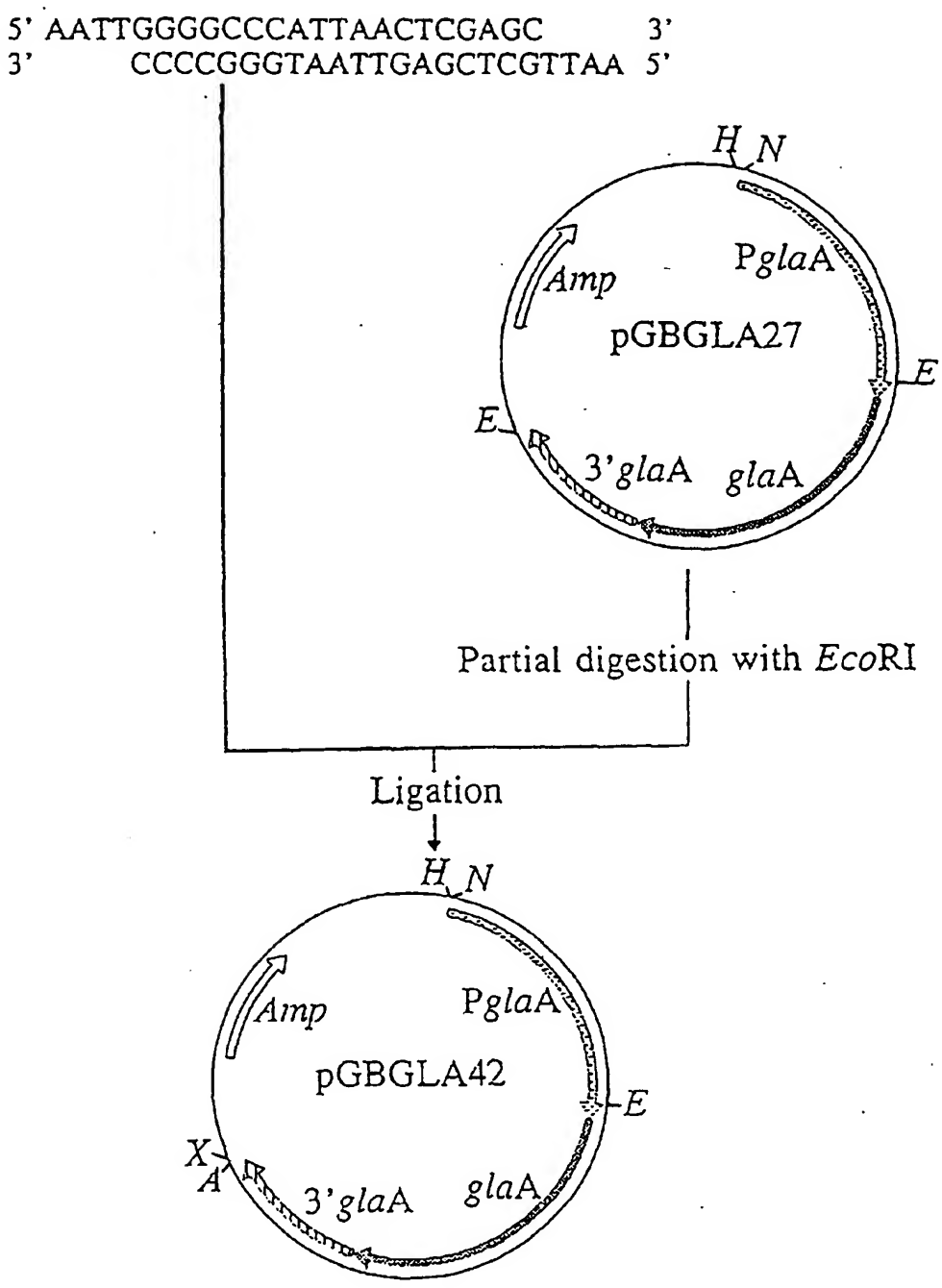

5' AATTGGGGCCCATTAACTCGAGC 3'
3' CCCCGGGTAATTGAGCTCGTTAA 5'
H N
Amp
PglaA
pGBGLA27
E
E
3'glaA
glaA
Partial digestion with EcoRI
Ligation
H N
Amp
PglaA
pGBGLA42
E
X
A
3'glaA
glaA

Fig. 16

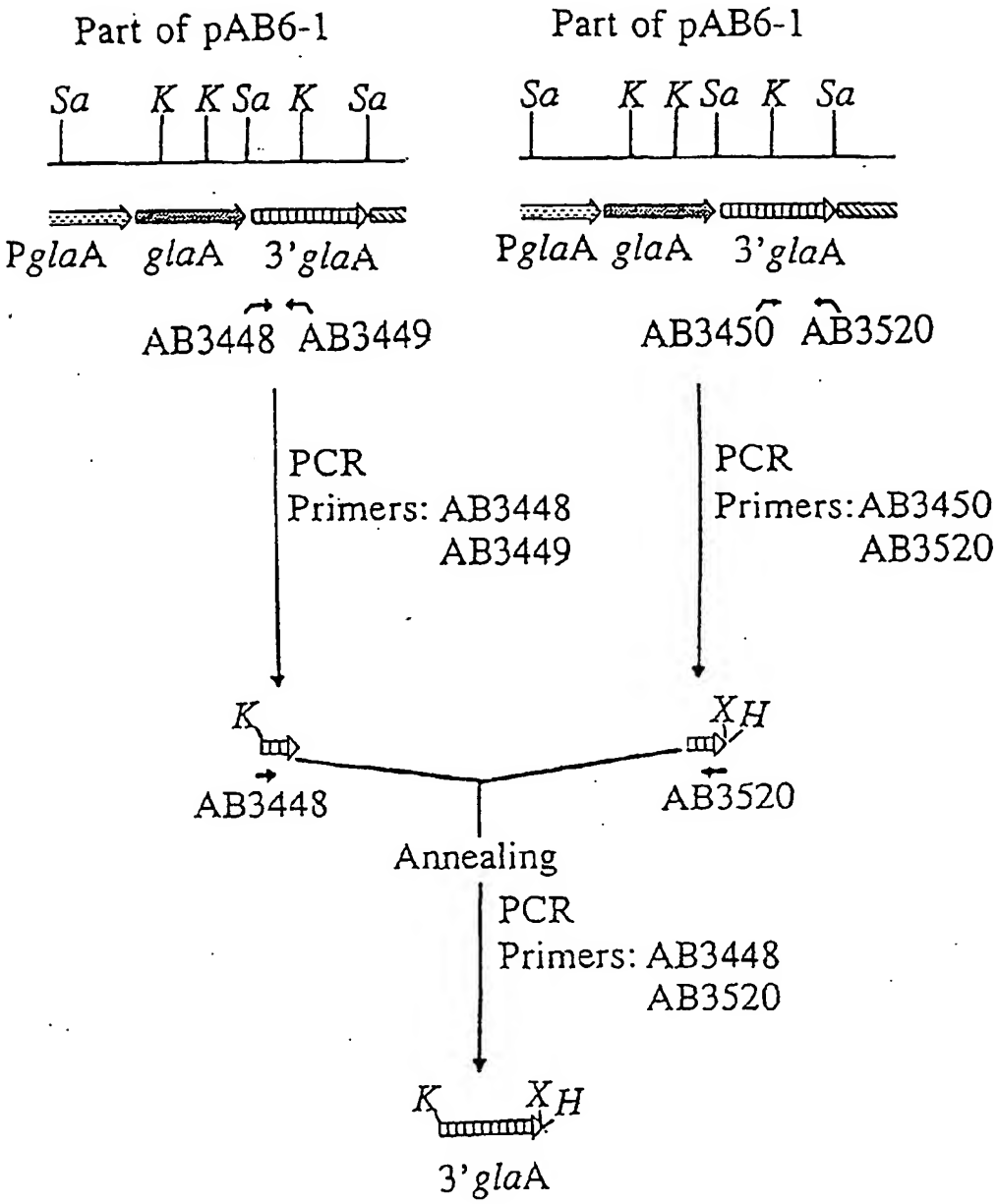

Part of pAB6-1
Sa K K Sa K Sa
PglaA glaA 3'glaA
AB3448 AB3449
PCR
Primers: AB3448
AB3449
Part of pAB6-1
Sa K K Sa K Sa
PglaA glaA 3'glaA
AB3450 AB3520
PCR
Primers: AB3450
AB3520
K
AB3448
XH
AB3520
Annealing
PCR
Primers: AB3448
AB3520
K XH
3'glaA

Fig. 17a

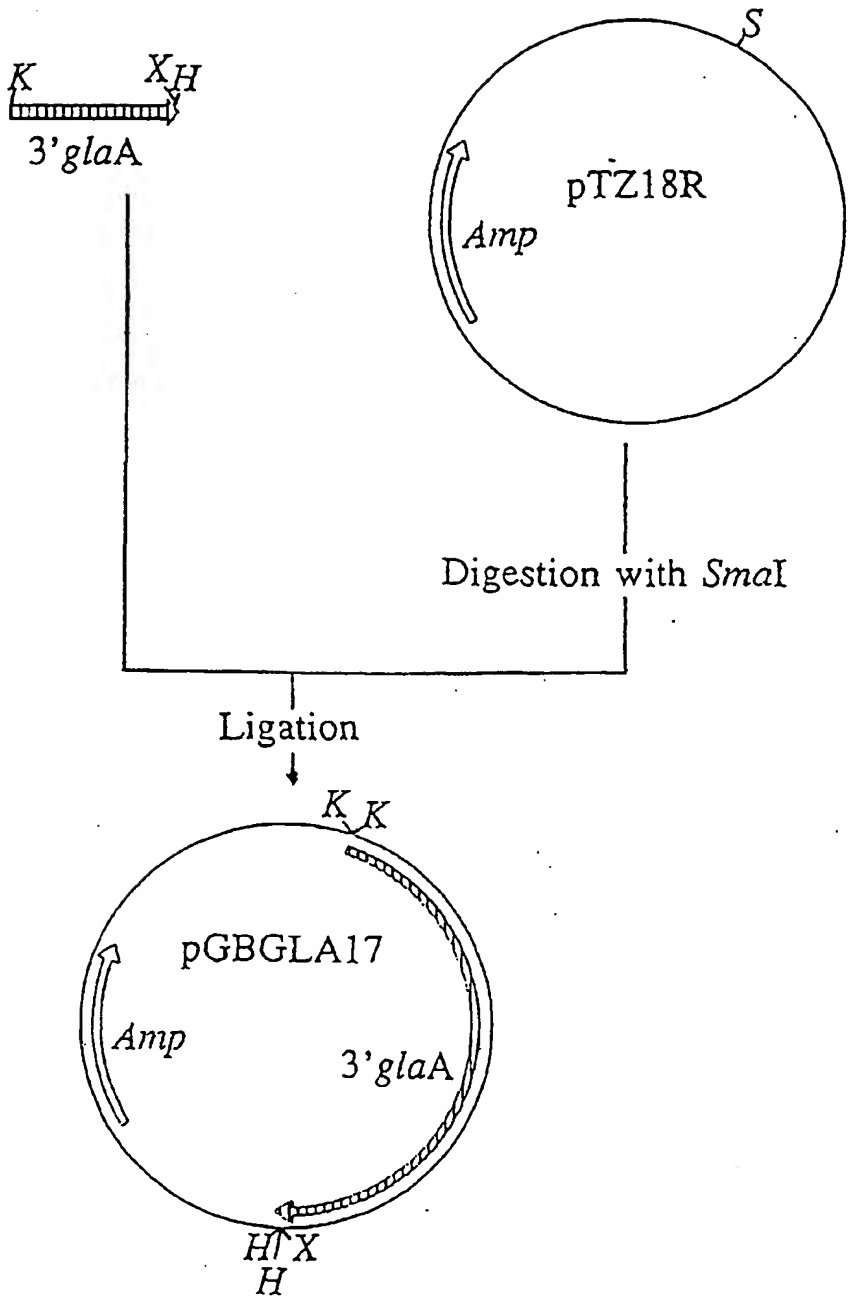

K
X_H
3'glaA
S
pTZ18R
Amp
Digestion with SmaI
Ligation
K K
pGBGLA17
Amp
3'glaA
H X
H

Fig. 17b

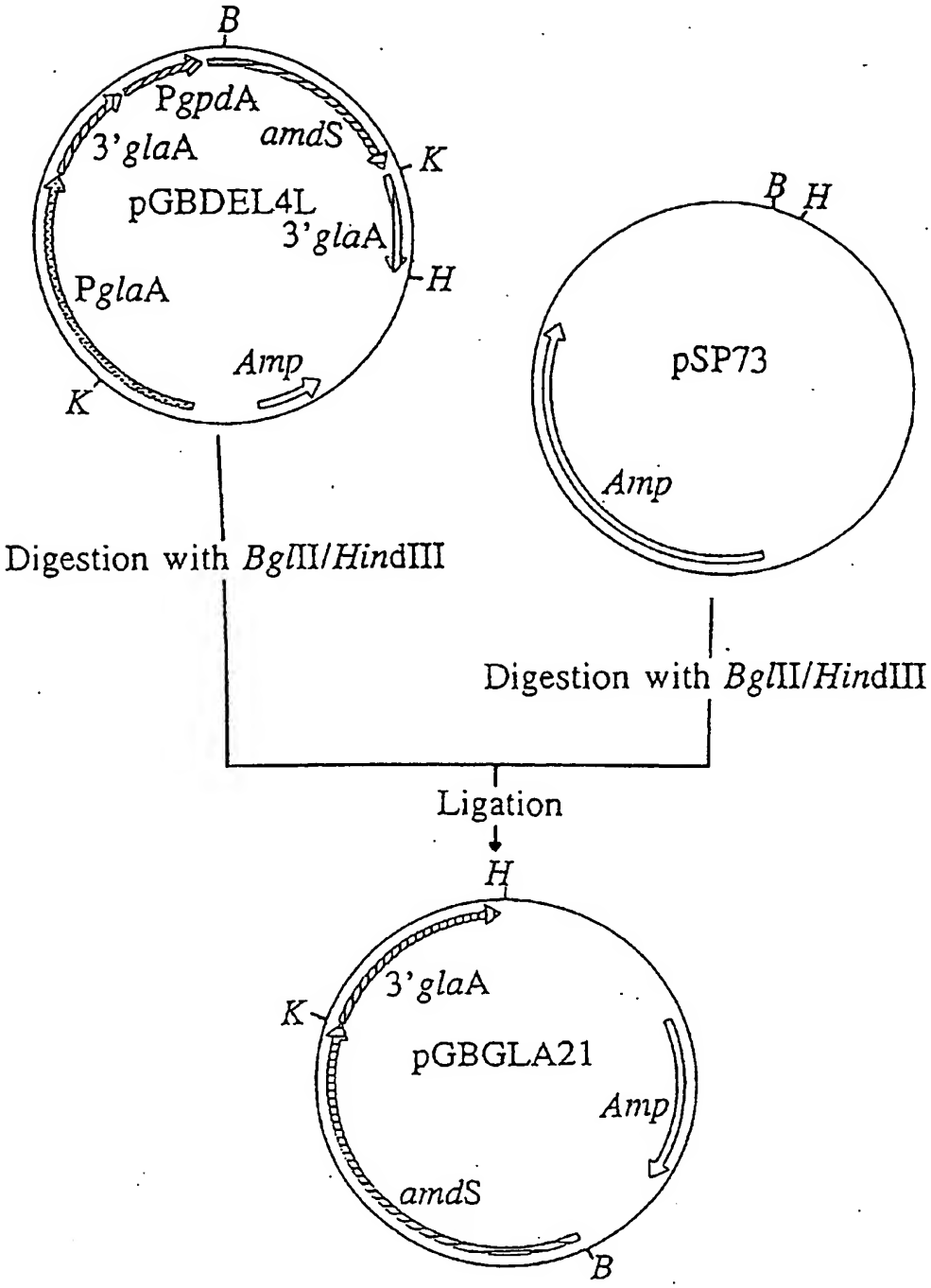

B
PgpdA
amdS
3'glaA
K
pGBDEL4L
3'glaA
H
PglaA
Amp
K
B H
pSP73
Amp
Digestion with BglII/HindIII
Digestion with BglII/HindIII
Ligation
H
3'glaA
K
pGBGLA21
Amp
amdS
B

Fig. 18

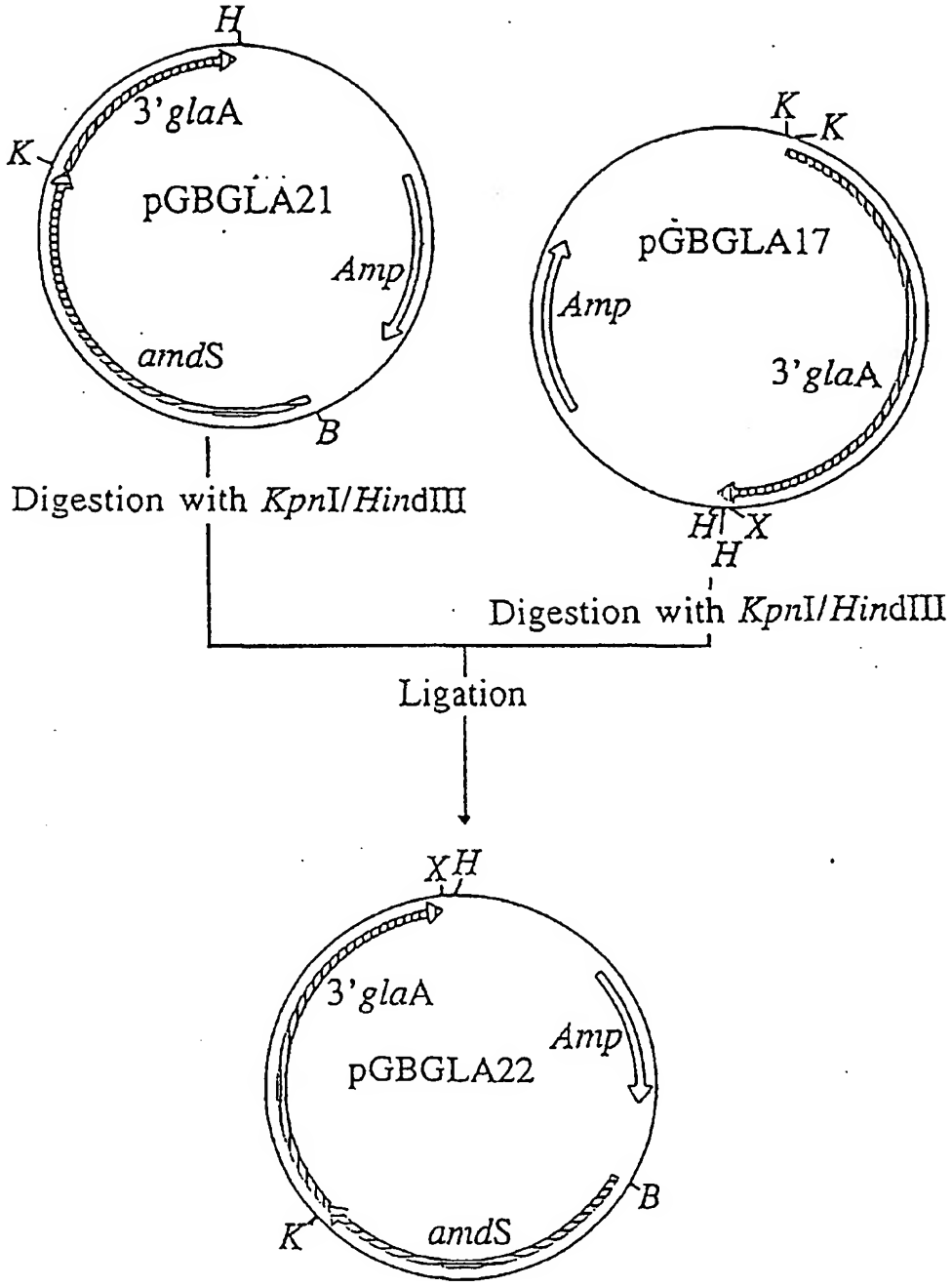

H
3'glaA
K
pGBGLA21
Amp
amdS
B
Digestion with KpnI/HindIII
K
K
pGBGLA17
Amp
3'glaA
H
X
H
Digestion with KpnI/HindIII
Ligation
XH
3'glaA
Amp
pGBGLA22
B
K
amdS

Fig. 19

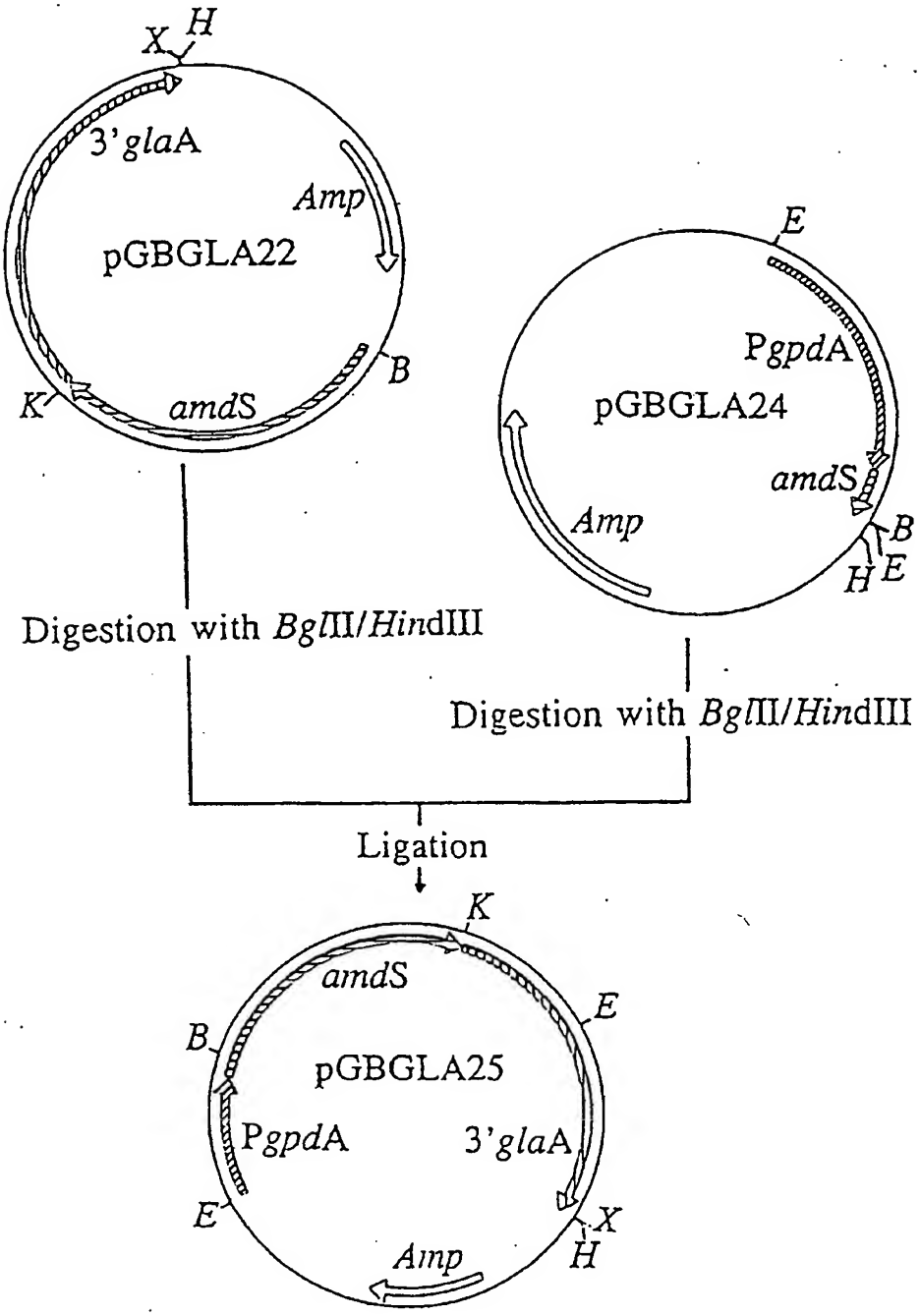

X
H
3'glaA
Amp
pGBGLA22
B
K
amdS
E
PgpdA
pGBGLA24
amdS
Amp
B
H
E
Digestion with BglII/HindIII
Digestion with BglII/HindIII
Ligation
K
amdS
E
B
pGBGLA25
PgpdA
3'glaA
E
X
H
Amp

Fig. 20

5' AATTGGGGCCCAGCGTCC 3'
3' CCCCGGGTCGCAGGTTAA 5'

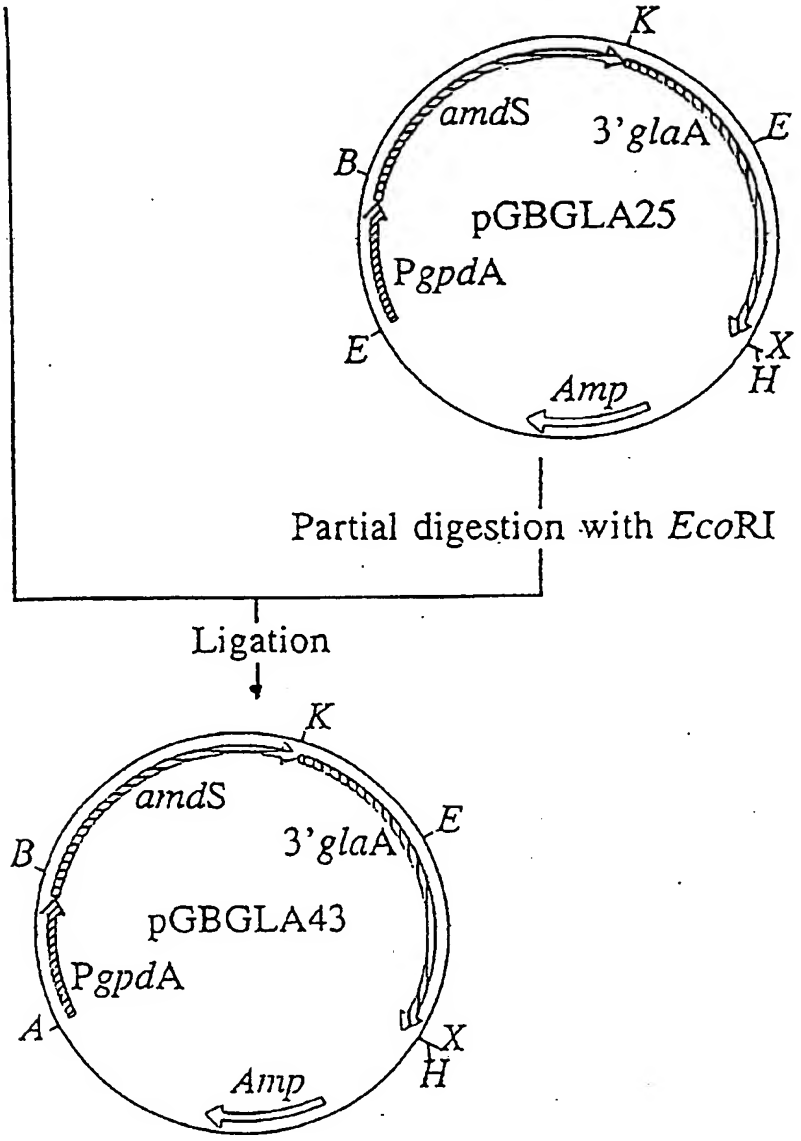


Fig. 21

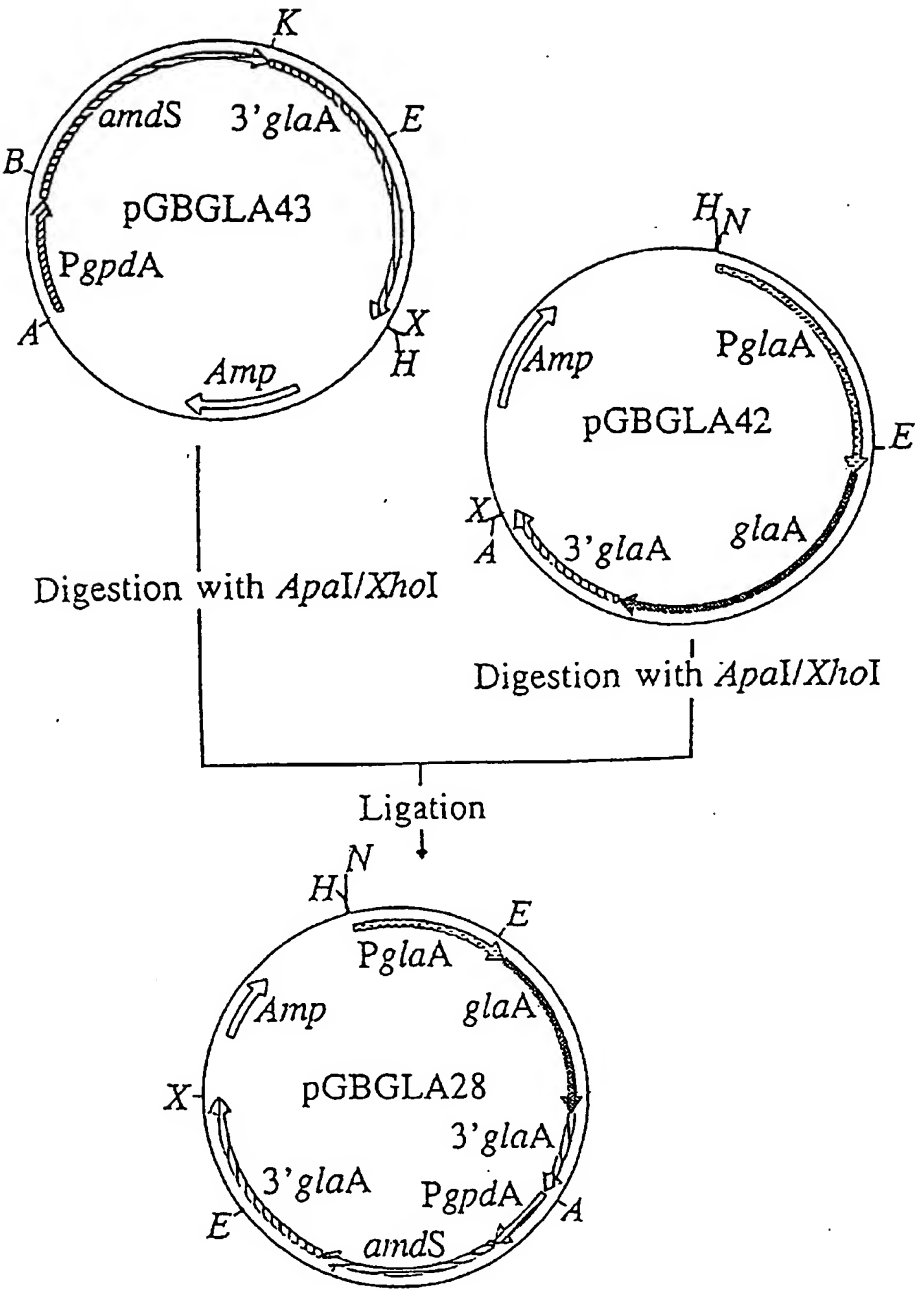

K
amdS
3'glaA
E
B
pGBGLA43
PgpdA
A
X
H
Amp
Digestion with ApaI/XhoI
H
N
Amp
PglaA
pGBGLA42
E
X
A
3'glaA
glaA
Digestion with ApaI/XhoI
Ligation
N
H
E
PglaA
Amp
glaA
X
pGBGLA28
3'glaA
3'glaA
PgpdA
A
E
amdS

Fig. 22

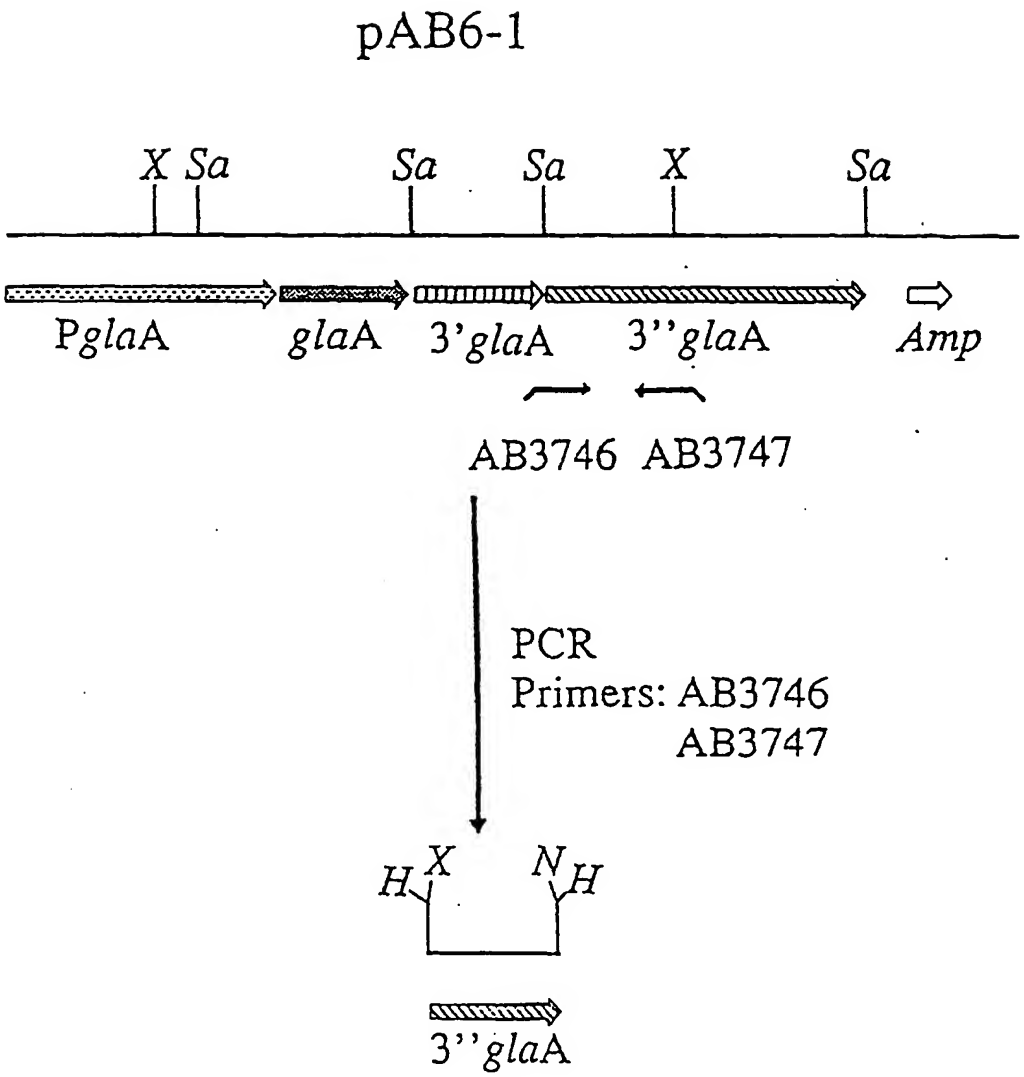

pAB6-1
X Sa
Sa
Sa
X
Sa
PglaA
glaA
3'glaA
3''glaA
Amp
AB3746 AB3747
PCR
Primers: AB3746
AB3747
H X
N H
3''glaA

Fig. 23a

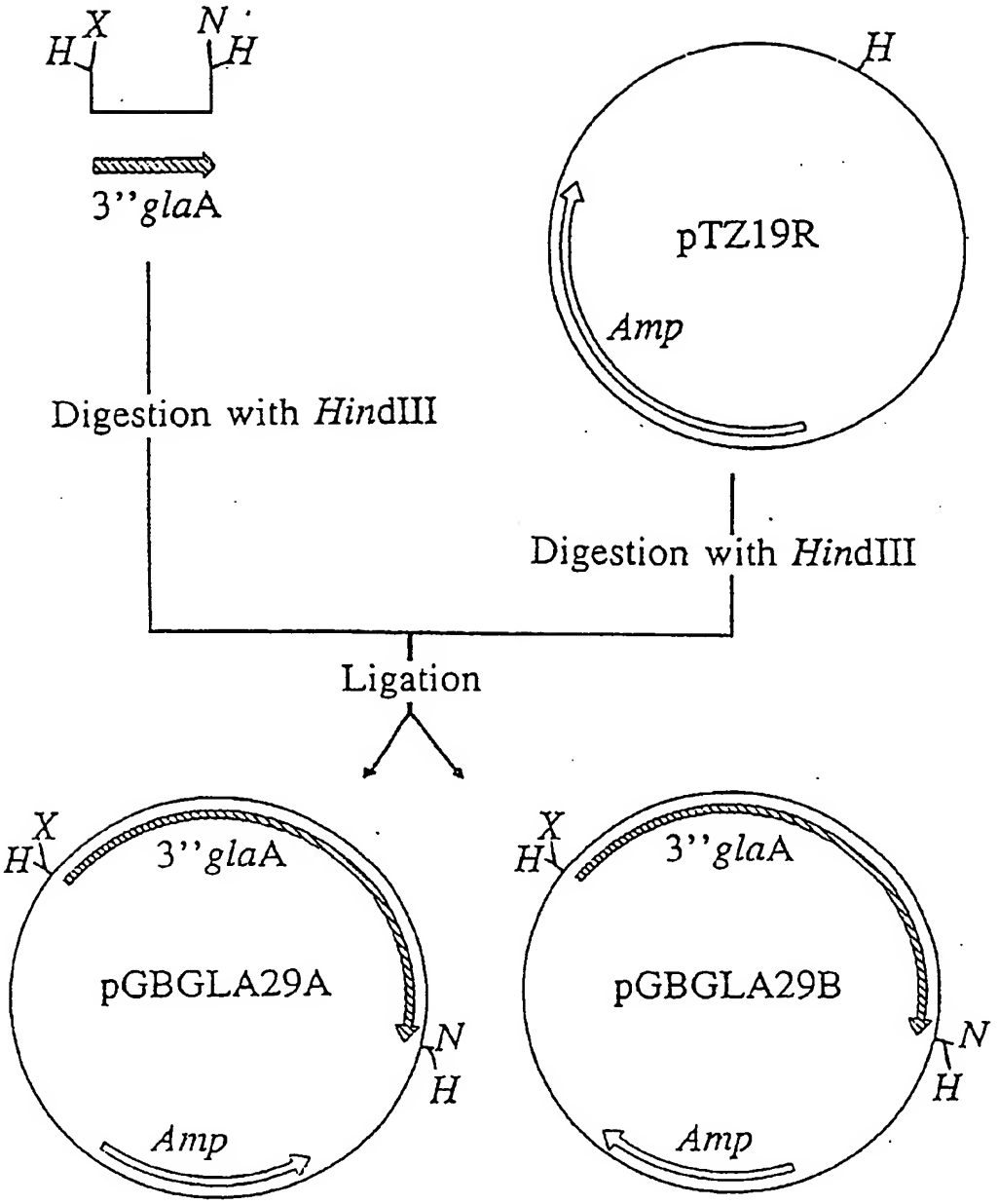

X
N
H
H
3''glaA
H
pTZ19R
Amp
Digestion with HindIII
Digestion with HindIII
Ligation
X
H
3''glaA
pGBGLA29A
N
H
Amp
X
H
3''glaA
pGBGLA29B
N
H
Amp

Fig. 23b

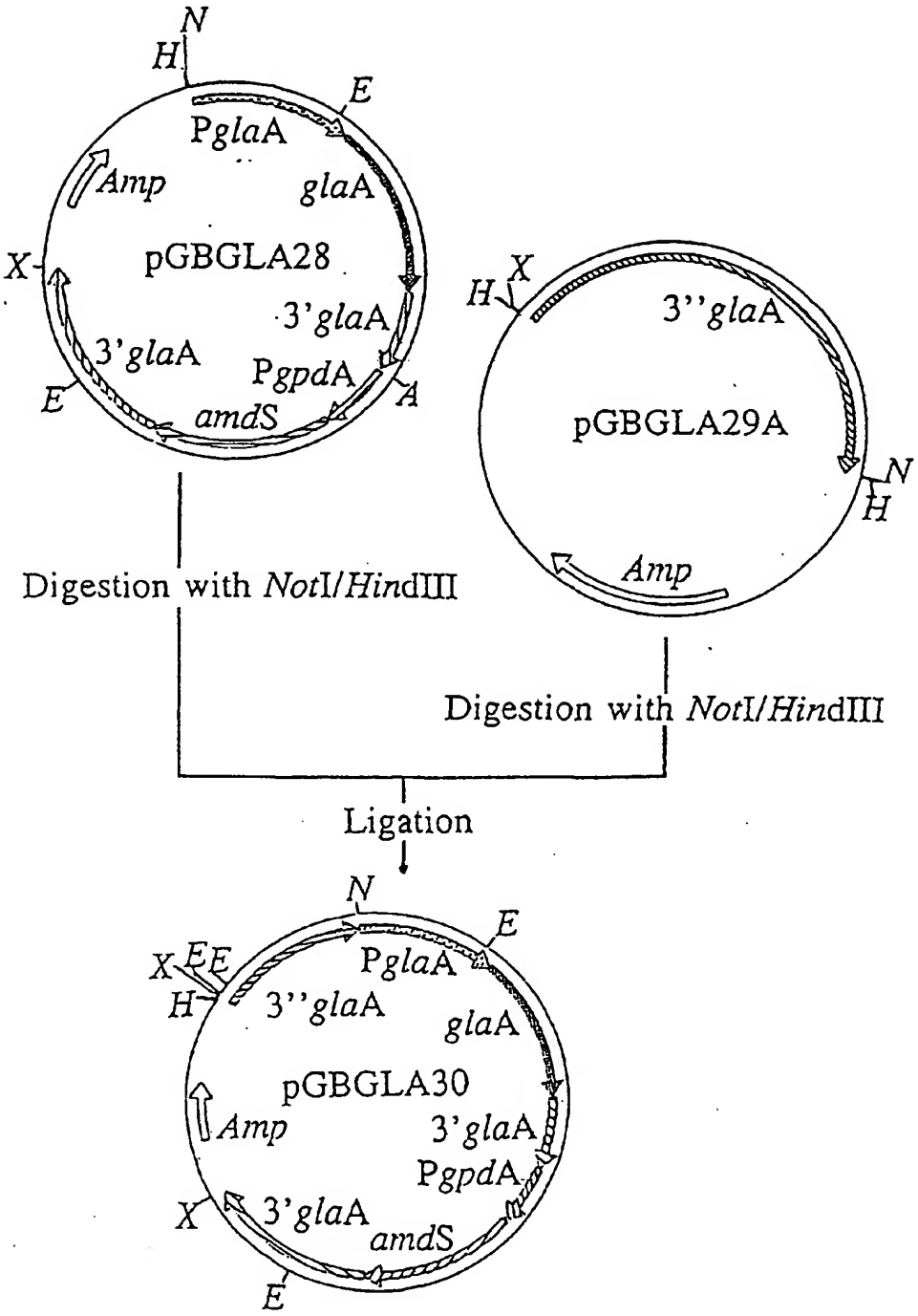

N
H
E
PglaA
Amp
glaA
pGBGLA28
X
3'glaA
3'glaA
PgpdA
E
amdS
A
X
H
3''glaA
pGBGLA29A
N
H
Amp
Digestion with NotI/HindIII
Digestion with NotI/HindIII
Ligation
N
E
X
E
E
PglaA
H
3''glaA
glaA
pGBGLA30
Amp
3'glaA
PgpdA
X
3'glaA
amdS
E

Fig. 24

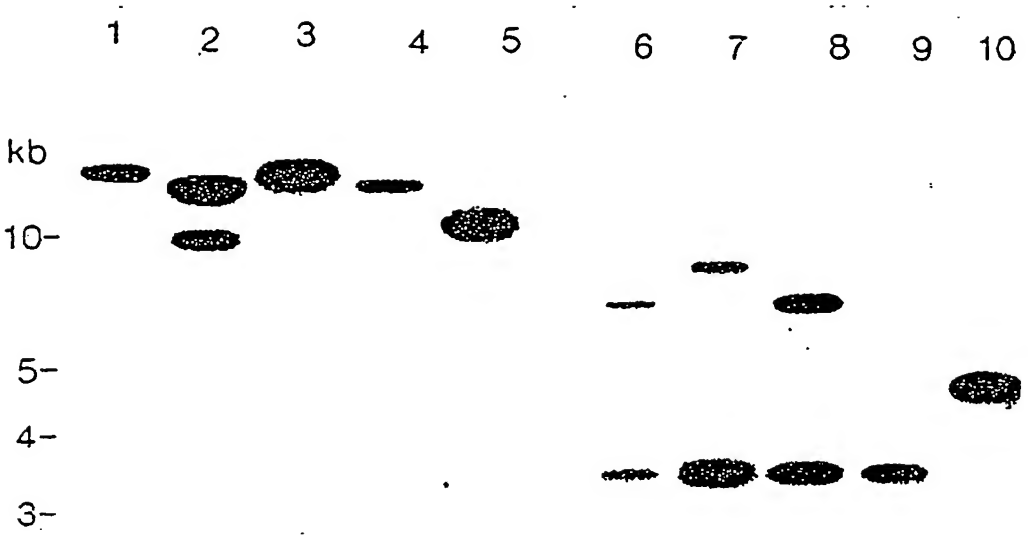


Fig. 25

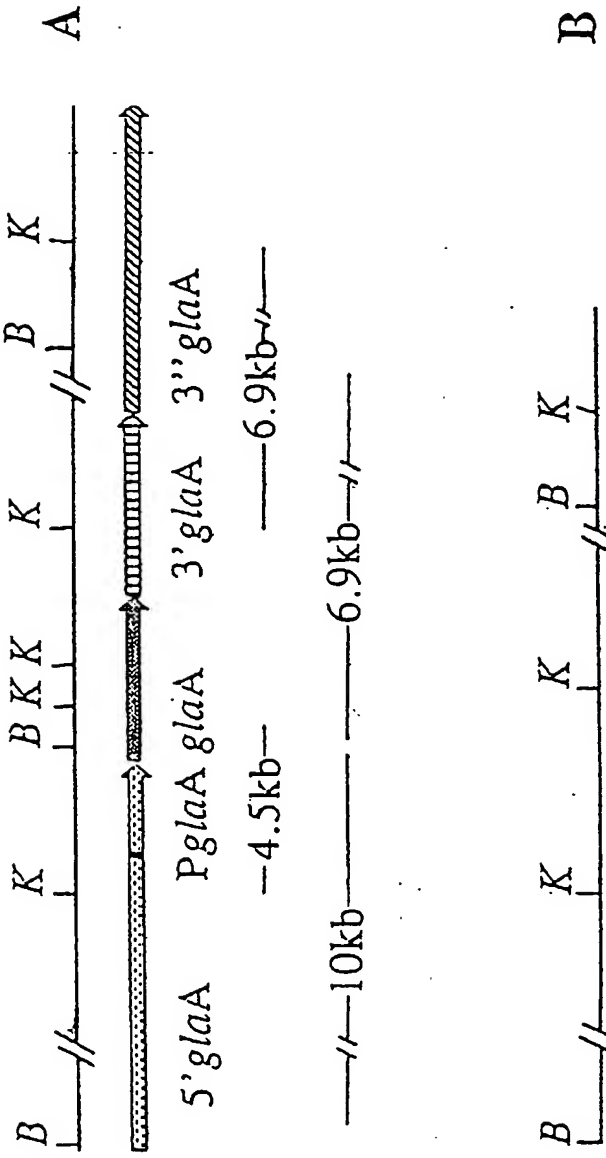

A
B
K
B K K
K
B
K
5'glaA
PglaA glaA
3'glaA
3''glaA
4.5kb
6.9kb
10kb
6.9kb
B
K
K
B
K
5'glaA
3'glaA
3''glaA
3.4kb
6.9 kb
13 kb
B

Fig. 26a

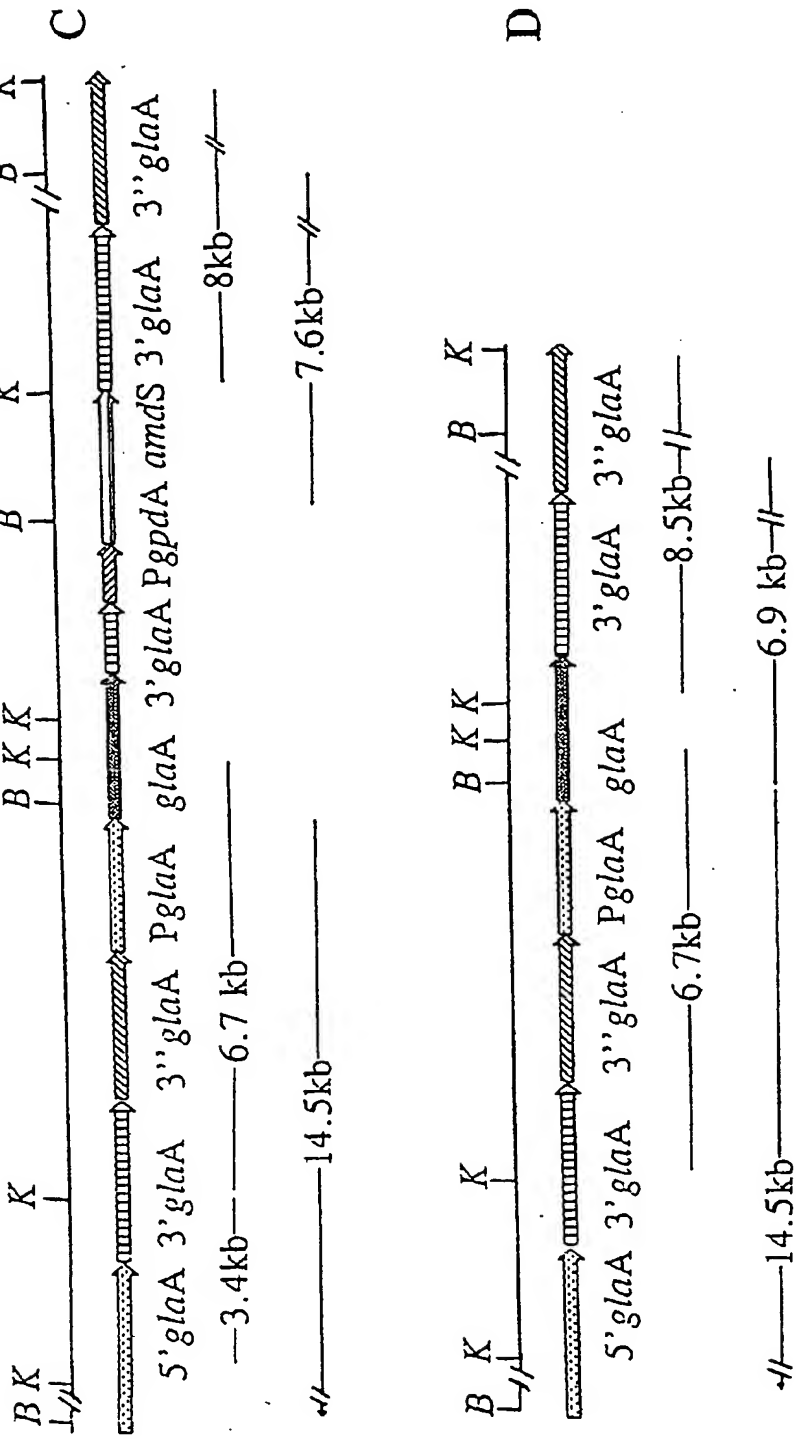

C
B K
K
B K K
B
K
B
K
5'glaA 3'glaA 3''glaA PglaA glaA 3'glaA PgpdA amdS 3'glaA 3''glaA
3.4kb
6.7 kb
8kb
14.5kb
7.6kb
D
B K
K
B K K
B
K
5'glaA 3'glaA 3''glaA PglaA glaA 3'glaA 3''glaA
6.7kb
8.5kb
14.5kb
6.9 kb

Fig. 26b

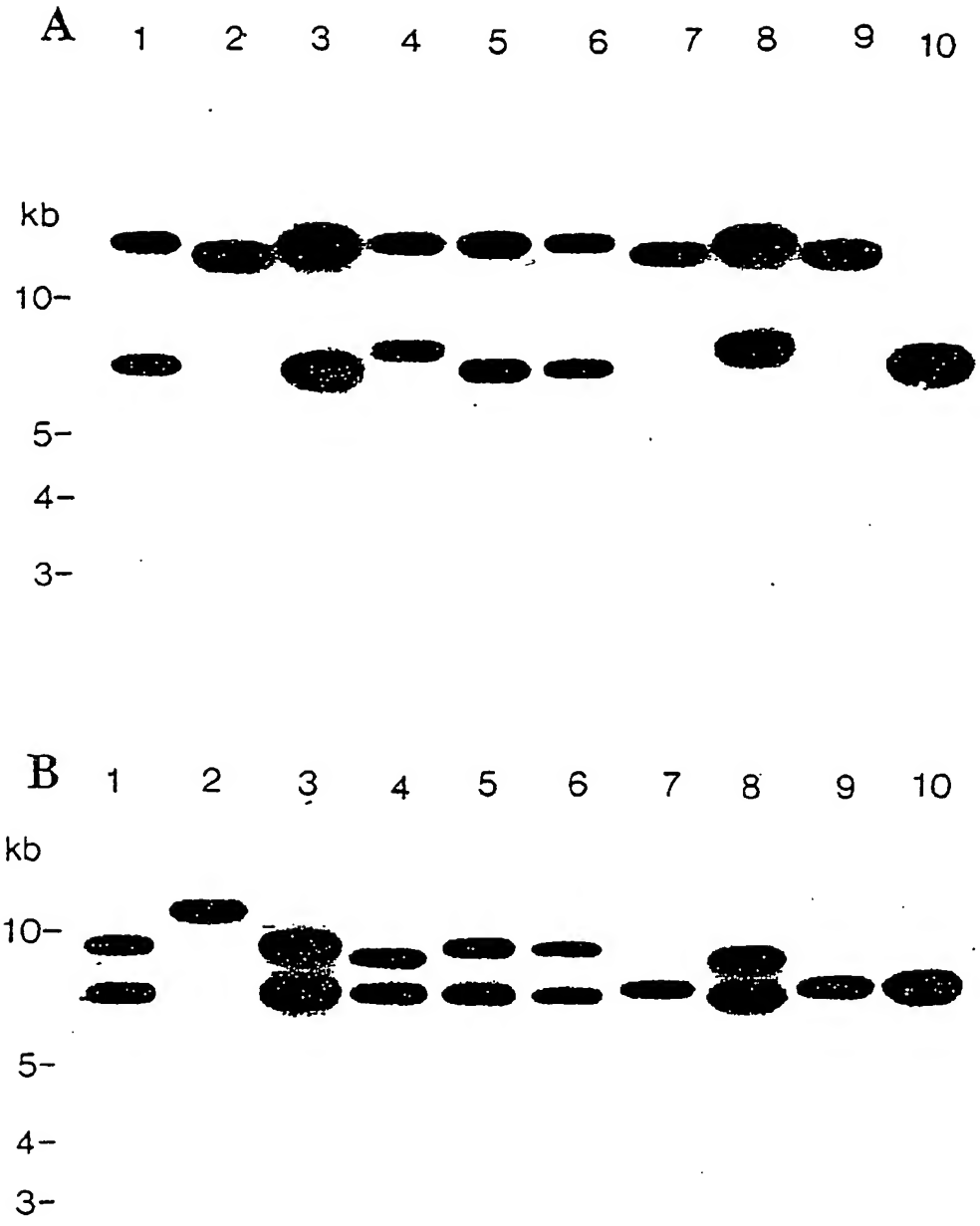


Fig. 27

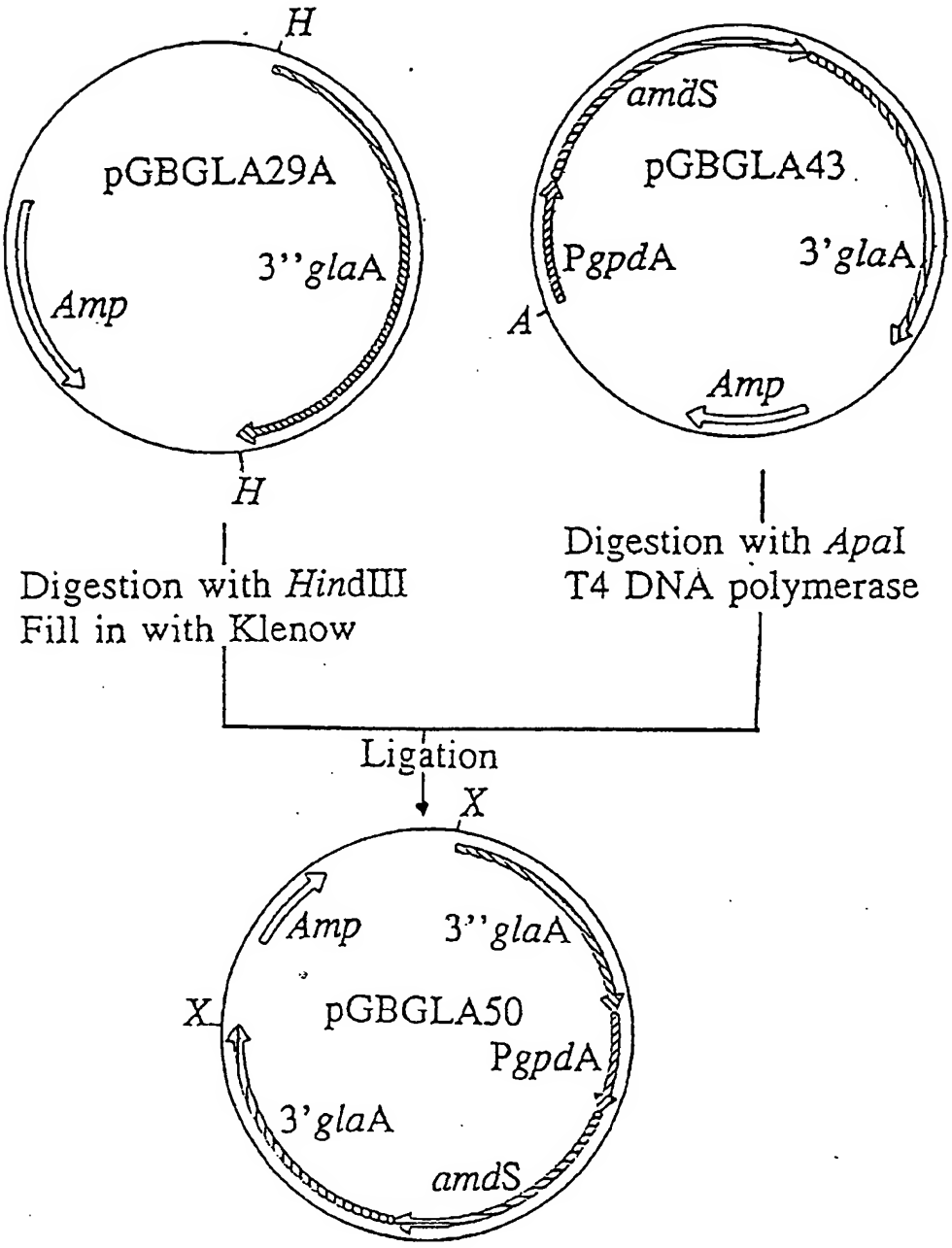

H
pGBGLA29A
3''glaA
Amp
H
amdS
pGBGLA43
PgpdA
3'glaA
A
Amp
Digestion with HindIII
Fill in with Klenow
Digestion with ApaI
T4 DNA polymerase
Ligation
X
Amp
3''glaA
X
pGBGLA50
PgpdA
3'glaA
amdS

Fig. 28

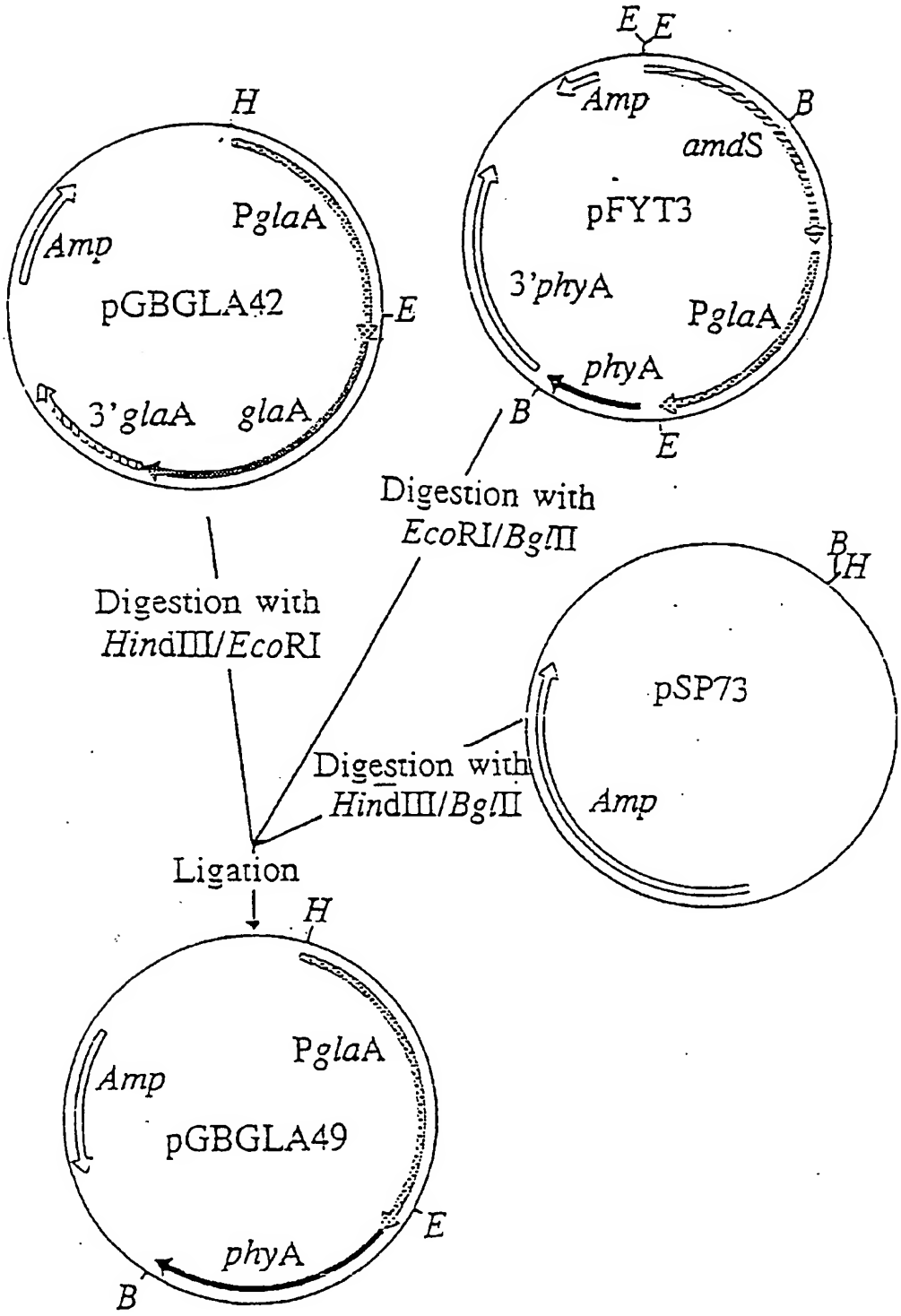

E
E
Amp
B
amdS
pFYT3
3'phyA
PglaA
phyA
B
E
H
PglaA
Amp
pGBGLA42
E
3'glaA
glaA
Digestion with
EcoRI/BglII
Digestion with
HindIII/EcoRI
B
H
pSP73
Amp
Digestion with
HindIII/BglII
Ligation
H
PglaA
Amp
pGBGLA49
E
phyA
B

Fig. 29

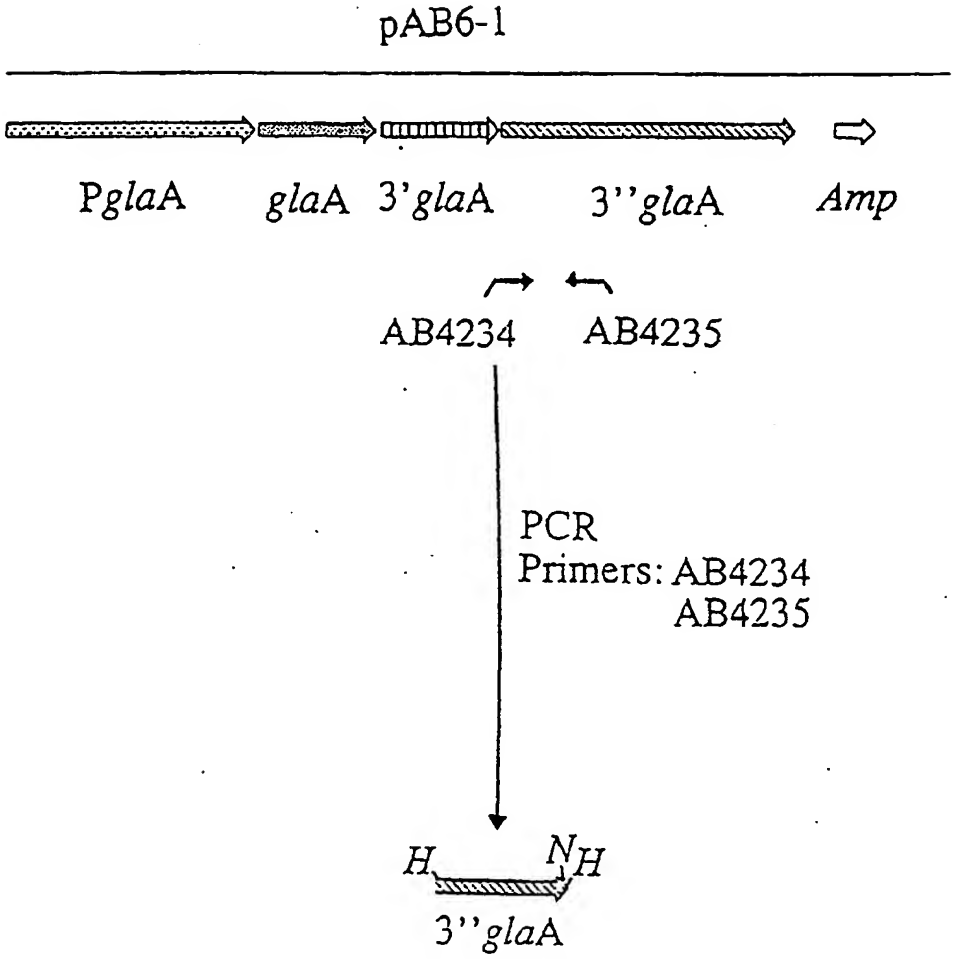

pAB6-1
PglaA
glaA
3'glaA
3''glaA
Amp
AB4234
AB4235
PCR
Primers: AB4234
AB4235
H
NH
3''glaA

Fig. 30a

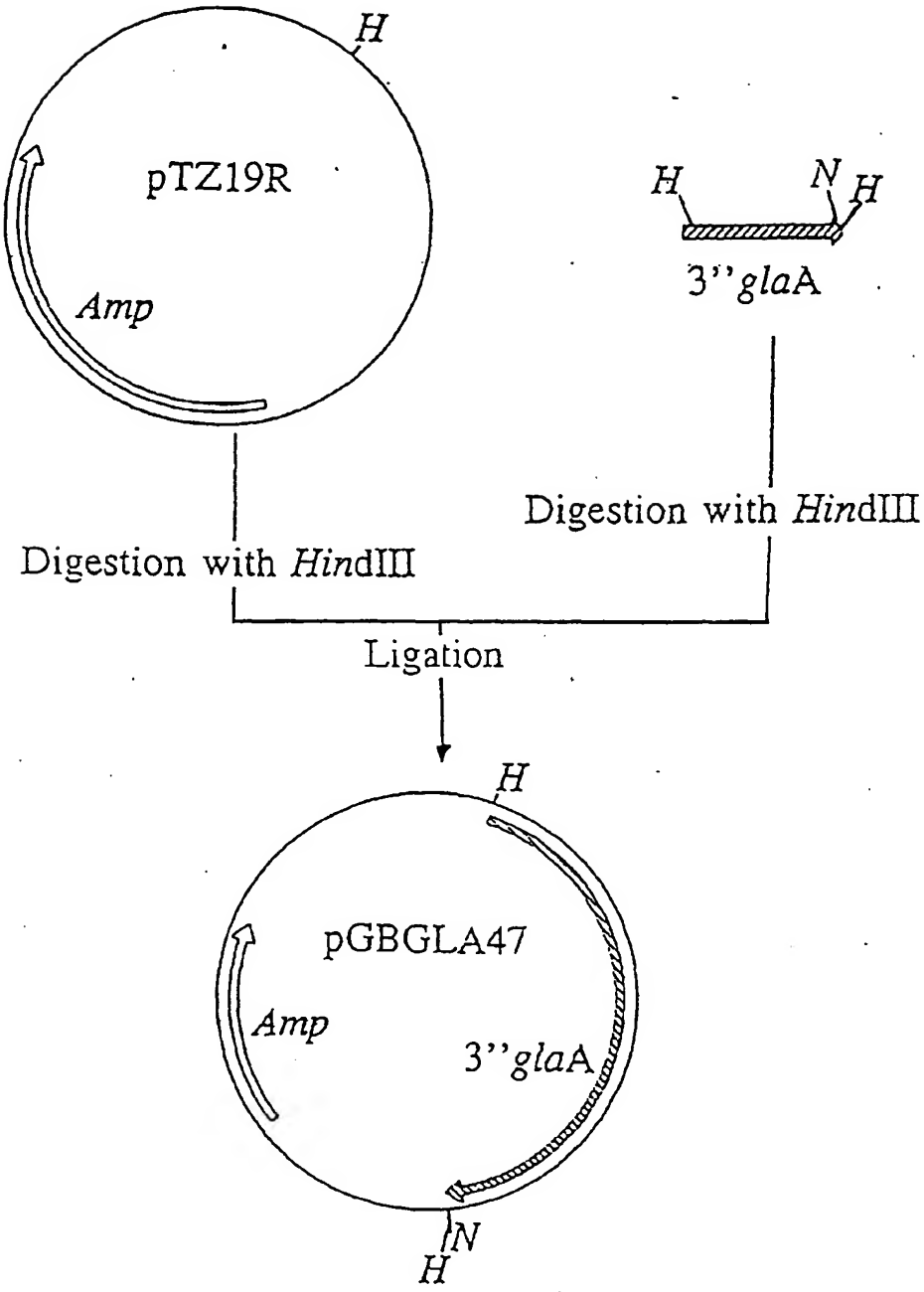

H
pTZ19R
Amp
H
N
H
3''glaA
Digestion with HindIII
Digestion with HindIII
Ligation
H
pGBGLA47
Amp
3''glaA
N
H

Fig. 30b

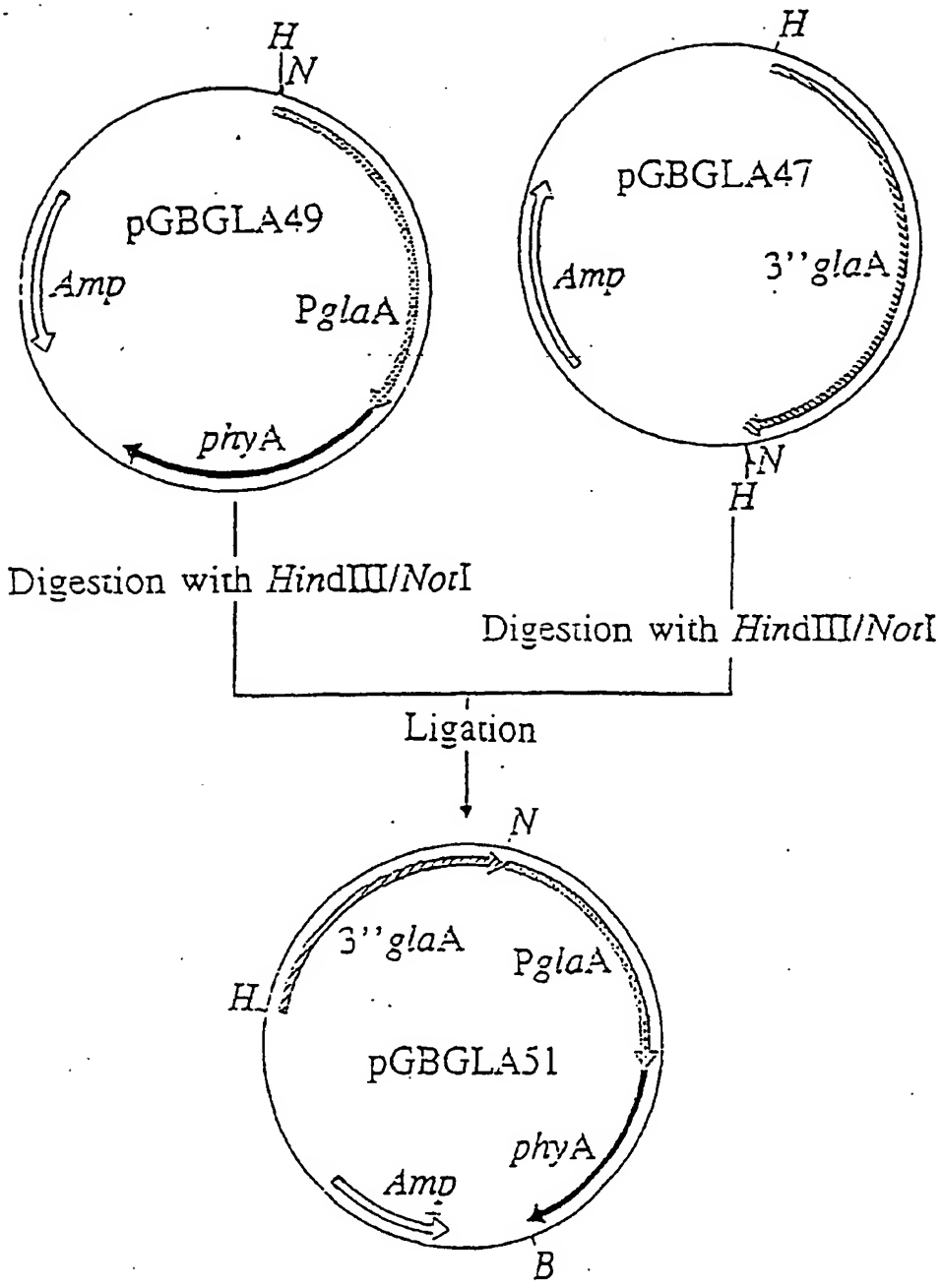

H
N
pGBGLA49
Amp
PglaA
phyA
H
pGBGLA47
Amp
3''glaA
N
H
Digestion with HindIII/NotI
Digestion with HindIII/NotI
Ligation
N
3''glaA
PglaA
H
pGBGLA51
phyA
Amp
B

Fig. 31

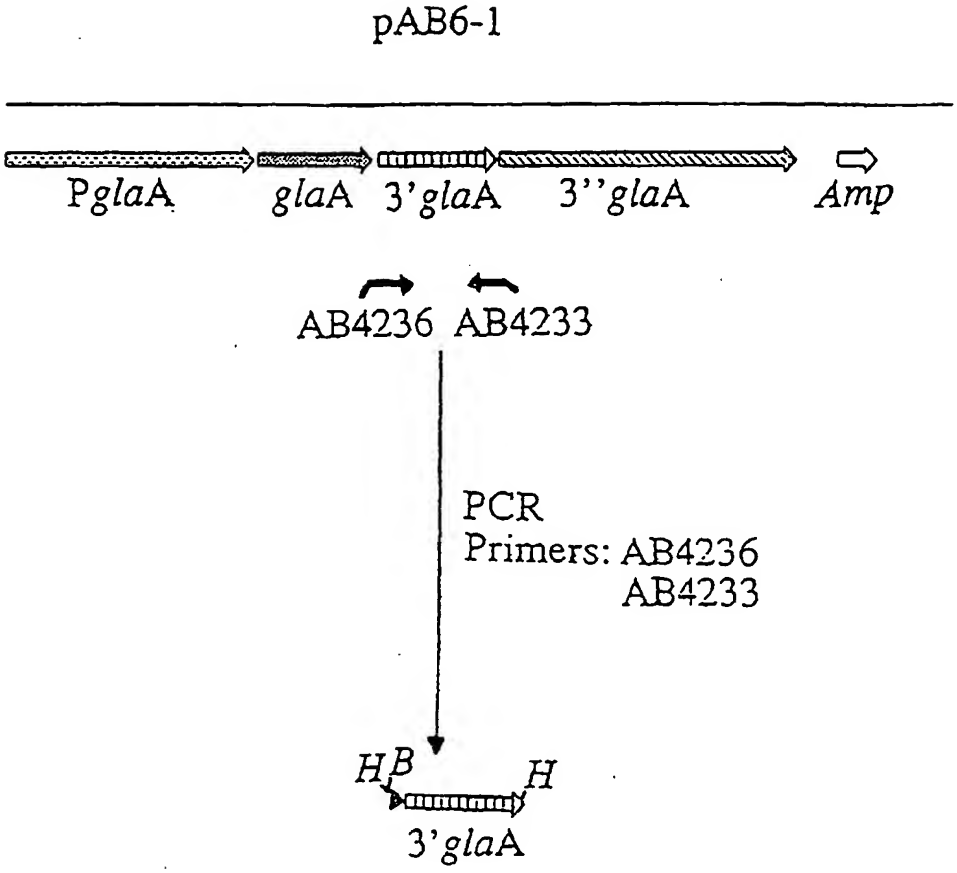

pAB6-1
PglaA
glaA
3'glaA
3''glaA
Amp
AB4236
AB4233
PCR
Primers: AB4236
AB4233
H
B
H
3'glaA

Fig. 32a

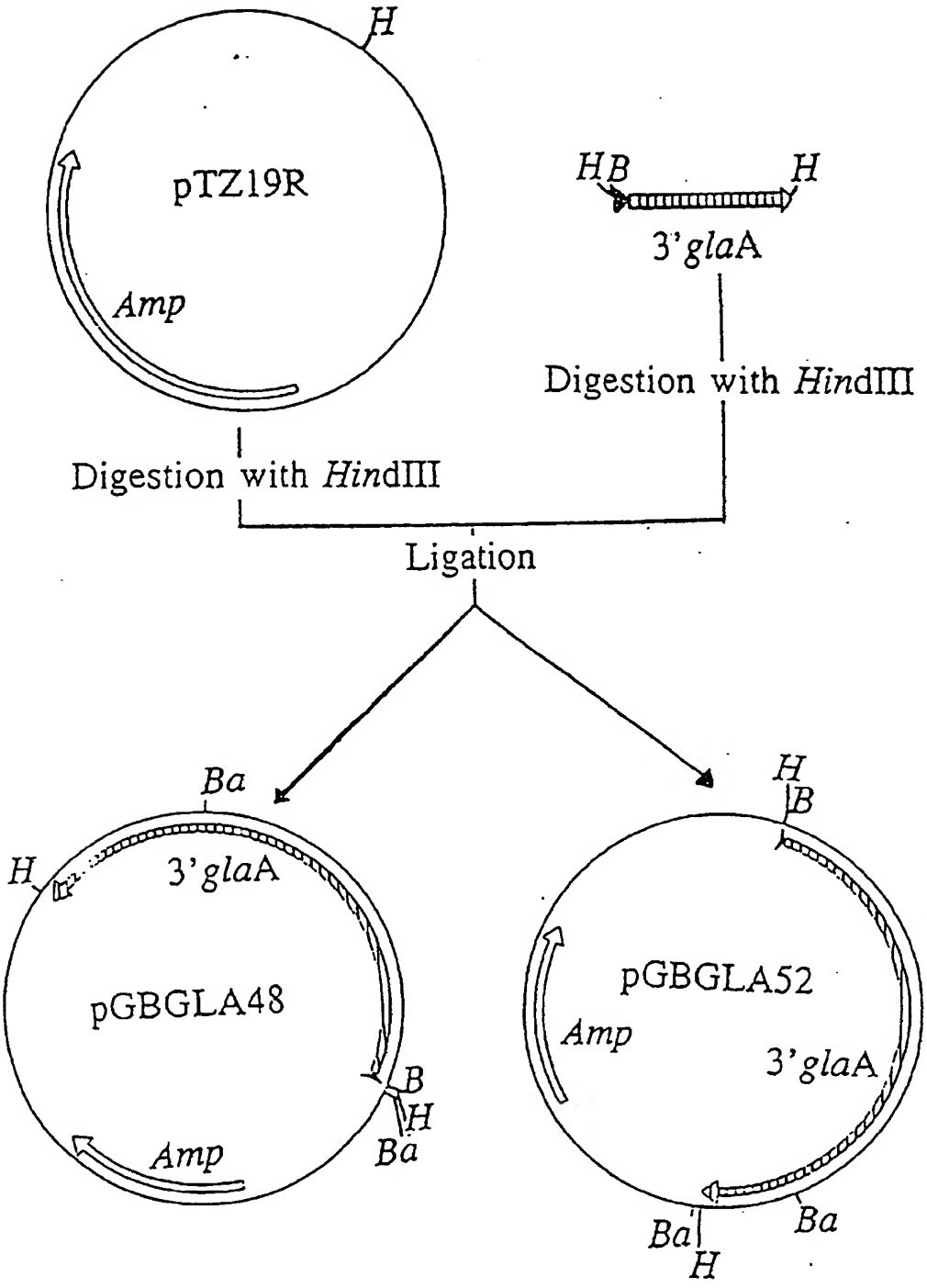

H
pTZ19R
Amp
HB
H
3'glaA
Digestion with HindIII
Digestion with HindIII
Ligation
Ba
H
3'glaA
pGBGLA48
B
H
Ba
Amp
H
B
pGBGLA52
Amp
3'glaA
Ba
Ba
H

Fig. 32b

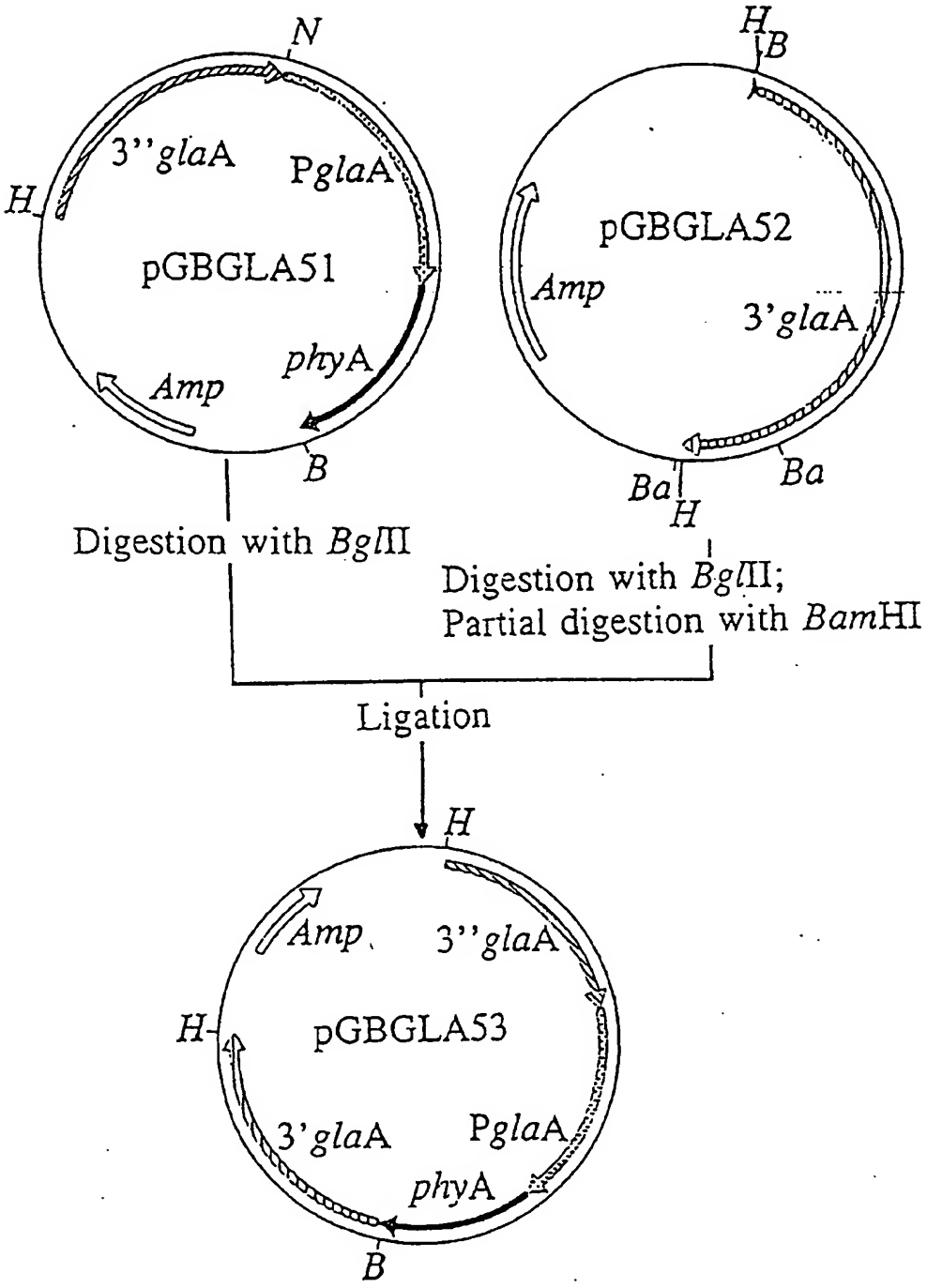

N
3''glaA
PglaA
H
pGBGLA51
phyA
Amp
B
H
B
pGBGLA52
Amp
3'glaA
Ba
H
Ba
Digestion with BglII
Digestion with BglII;
Partial digestion with BamHI
Ligation
H
Amp
3''glaA
H
pGBGLA53
3'glaA
PglaA
phyA
B

Fig. 33